GENETIC AND PHENOTYPIC TRAITS IN POPULATIONS COMPRISING DIPLOID AND POLYPLOID HYBRID WATER FROGS (ANURA, *PELOPHYLAX ESCULENTUS*)

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“Those forms which possess in some considerable degree the character of species, but which are so closely similar to some other forms, or are so closely linked to them by intermediate gradations, that naturalists do not like to rank them as distinct species, are in several respects the most important to us.”

Charles Darwin (1859)*

*From: The origin of species by means of natural selection or the preservation of favoured races in the struggle of life

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Summary

The edible frog (*Pelophylax esculentus*, genotypes LR, LLR or LRR) is a natural hybrid between the pool frog (*P. lessonae*, genotype LL) and the marsh frog (*P. ridibundus*, genotype RR). Diploid hybrids (LR) reproduce by hybridogenesis, where one part of the hybrid's parental genome (either the L or the R chromosome set) is excluded during gametogenesis and the other part is clonally transmitted into haploid gametes. Recombination between the L and R genome within the hybrid is usually not possible. Therefore, repeated clonal transmission of one part of the genome leads to the accumulation of deleterious mutations which normally renders offspring from inter-hybrid crossings within the same population unviable. In order to produce viable offspring, the hybrid is thus forced to mate with the parental species whose part of the genome was excluded. This reproductive dependence has led to several forms of mixed hybrid-parental population systems.

In some populations, *P. esculentus* can produce both haploid (L or R) and diploid gametes, LR gametes usually coming only from LR individuals. The fusion of diploid with haploid gametes results in triploid hybrids of the genotypes LLR and LRR, which exclude the single copy genome and produce only haploid gametes of the other genome (LLR produce L, LRR produce R gametes). Thus, in a perpetuating way, diploid and triploid hybrids are generated and can successfully reproduce with each other, independent of the presence of the parental species. The reproductive independence of these so-called mixed-ploidy systems is due to the fact that triploids recombine the part of their genome which is present in a double copy and thus prevent the accumulation of deleterious alleles in the genetic pool of the population.

Both the mixed hybrid-parental and the mixed-ploidy systems have been studied over the last decades in several aspects and geographic regions, but due to the patchy geographic distribution of mixed-ploidy systems in Europe, a comprehensive and comparative population genetic overview across a larger area has been lacking. Furthermore, population genetic and phenotypic differences between hybrid *P. esculentus* from mixed-parental and mixed-ploidy systems are vastly unknown, despite potentially different selection regimes between population types and geographic regions. The objectives of my thesis were thus: a) to compare mixed-ploidy populations from different European areas in a genealogical approach and to find out whether these patchily distributed populations are of independent

origin, b) to examine the gamete production patterns between two supposedly different mixed-ploidy breeding systems, c) to study bioacoustic characteristics of male advertisement calls across a number of geographically distant mixed-ploidy populations and compare them with hybrids from mixed hybrid-parental systems, and d) to examine the spatial movement and spacing behavior of male frogs within and between mixed-ploidy and mixed hybrid-parental systems and relate potential differences to male genotype, male morphology and pond characteristics.

In **chapter one**, I used microsatellite DNA and mitochondrial DNA analysis to obtain population genetic parameters for more than 2000 samples from 72 localities across Northern, Central and Eastern Europe. The results from this study showed that genetic diversity among populations based on microsatellites is structured by geographic latitude and longitude, the presence of parental genotypes (*P. lessonae* and *P. ridibundus*) and population type. Most mixed-ploidy populations from Central and East-Central Europe did not genetically differ substantially, but some populations from Ukraine showed a distinctively different genetic profile. This was confirmed by the novel finding of unusual types of mitochondrial DNA in specimens from there. My findings suggest an independent origin of polyploid water frogs from this area, which I discuss with reference to postglacial re-colonization scenarios in Europe.

Chapter two presents a collaborative study with Nicolas Pruvost, where we used microsatellite DNA analyses and crossing experiments to compare five populations of different population structures. Indices of heterozygosity and genetic differentiation were used to depict the genetic interactions between the different genotypes (LL, LLR, LR, LRR and RR). The results from this study allowed us to define and differentiate between different breeding systems and propose an evolutionary scenario for the occurrence and maintenance of an alternative mixed-ploidy population type in Central Europe.

In **chapter three** I studied the bioacoustic properties of male advertisement calls within and between mixed-ploidy and mixed hybrid-parental populations. From field recordings I derived five call parameters which all showed a genomic dosage effect, i.e. they either decreased or increased with the L/R ratio among genotypes in the order LL-LLR-LR-LRR-RR. Two of the five call parameters were also affected by the population system. Effect sizes decreased from genotype through population system to geographic location of the population. Calls of diploid (LR) hybrids varied

between population systems, depending on whether they belonged to a system that required a sexual host for successful reproduction, or not.

In **chapter four** I tested within three ponds whether male spatial movement and spacing behavior during the breeding season differs between genotypes. Furthermore, I related spatial parameters to male body size and condition, and to the observed frequency of amplexus by individual males. As a result, I found that neither genotype nor size nor condition affected spatial movement patterns and that differences are most likely to be explained by population density. The frequency of amplexus events among genotypes corresponded to the observed male genotype distribution in the two mixed-ploidy ponds, and slightly favored *P. lessonae* males in the mixed hybrid-parental population in the other pond.

Chapter five represents a collaborative study with Anke Stöhr on ranavirus infection in wild populations of European water frogs. The study combines a case study of a ranavirus outbreak among captive water frogs with the description of a novel ranavirus and its phylogenetic classification.

In conclusion, chapters one to four of my thesis allow a better understanding of the diversity, distribution and differentiation of *P. esculentus* populations in terms of genetic and phenotypic characteristics. I argue that there is no such thing as a “common” water frog hybrid, but rather that hybrid populations are so diverse that they represent evolutionary significant units which deserve the same respect and attention as other “true” anuran species.

Zusammenfassung

Der Teichfrosch (*Pelophylax esculentus*, Genotypen LR, LLR oder LRR) ist ein natürlicher Hybrid zwischen dem Kleinen Wasserfrosch (*P. lessonae*, Genotyp LL) und dem Seefrosch (*P. ridibundus*, Genotyp RR). In der rein diploiden Form (LR) pflanzt sich der Hybrid via Hybridogenese fort, bei welcher ein Teil des elterlichen Genoms (entweder der L- oder der R-spezifische Teil) während der Gametogenese eliminiert und der verbleibende Teil klonal auf haploide Keimzellen übertragen wird. Rekombination zwischen dem L- und R-spezifischen Teil des Genoms ist in der Regel nicht möglich, weshalb es durch die wiederholte klonale Weitergabe innerhalb des Genoms zu einer Ansammlung schädlicher Mutationen kommt. Bei der Verpaarung zweiter Hybriden aus derselben Population sind deren Nachkommen durch die Kombination zweier klonaler Genome daher nicht überlebensfähig. Um lebensfähige Nachkommen zu zeugen, müssen sich die Hybriden mit der jeweiligen Elternart rückkreuzen, deren Teil des Hybridgenoms während der Gametogenese verloren ging. Aus dieser fortplanzungsbedingten Abhängigkeit heraus entwickelten sich verschiedene Formen eines gemischten Populationssystems aus Hybriden und Elternarten.

In einigen Populationen produziert *P. esculentus* sowohl haploide (L oder R) als auch diploide Keimzellen (LR-Gameten, welche normalerweise nur von Individuen des Genotyps LR gebildet werden). Aus der Verschmelzung von diploiden und haploiden Keimzellen entstehen triploide Hybriden der Genotypen LLR und LRR, welche ihrerseits bei der Gametogenese jenen Genomteil ausschliessen, der nur in einzelner Ausführung vorliegt, und aus dem doppelt vorhandenen Genomteil haploide Gameten bilden (so produziert der Genotyp LLR haploide L-Gameten und der Genotyp LRR haploide R-Gameten). Dadurch entstehen über die Generationen hinweg abwechselnd diploide und triploide Hybride, welche sich unabhängig von der Anwesenheit der Elternarten erfolgreich miteinander fortpflanzen. Die reproduktive Unabhängigkeit dieser sogenannten gemischt-ploiden Systeme wird dadurch ermöglicht, dass die in den triploiden Fröschen doppelt vorhandenen Genomteile rekombiniert werden können, wodurch verhindert wird, dass sich in den Genomen zu viele schädliche Mutationen ansammeln.

Sowohl die gemischten Systeme aus Hybriden und Elternarten als auch gemischt-ploide Systeme wurden in den letzten Jahrzehnten unter verschiedenen

Aspekten und in unterschiedlichen geographischen Regionen untersucht. Jedoch fehlte bislang aufgrund der ungleichmässigen Verbreitung gemischt-ploider Populationssysteme in Europa ein umfassender und vergleichender populationsgenetischer Überblick im grösseren geographischen Masstab. Ferner sind die populationsgenetischen und phänotypischen Unterschiede zwischen Hybriden aus Elternarten-Populationen und gemischt-ploiden Populationen bisher noch weitgehend unbekannt, obwohl zwischen den unterschiedlichen Systemen, als auch zwischen geographischen Regionen, potentiell unterschiedliche Selektionsbedingungen herrschen. Die Zielsetzungen meiner Arbeit waren daher: a) gemischt-ploide Populationen aus unterschiedlichen Gegenden Europas genealogisch zu untersuchen und herauszufinden, ob deren ungleichmässige Verteilung auf eine unabhängige Entstehungsgeschichte zurückzuführen ist, b) zwei augenscheinlich unterschiedliche gemischt-ploide Fortpflanzungssysteme auf Unterschiede in der Keimzellenproduktion zu untersuchen, c) die bioakustischen Eigenschaften männlicher Paarungsrufe einer Anzahl gemischt-ploider Populationen unterschiedlicher geographischer Herkunft zu analysieren und mit den Eigenschaften der Paarungsrufe aus gemischten Hybrid-Elternarten-Populationen zu vergleichen, und d) die räumlichen Bewegungsmuster und das Distanzverhalten zwischen Männchen verschiedener Genotypen aus gemischt-ploiden und gemischten Hybrid-Elternart-Populationen zu untersuchen und potentielle Unterschiede auf Zusammenhänge mit phänotypischen Eigenschaften der Männchen sowie mit Eigenschaften der untersuchten Teiche zu testen.

In **Kapitel eins** untersuchte ich durch Analysen von Mikrosatelliten-DNA und mitochondrialer DNA populationsgenetische Parameter für mehr als 2000 Gewebeproben, welche aus 72 Lokalitäten in Nord-, Mittel- und Osteuropa stammten. Die Ergebnisse dieser Studie zeigten, dass die auf der Mikrosatelliten-Analyse basierende genetische Diversität von der geographischen Lage, dem Vorhandensein der Elternarten *P. lessonae* und *P. ridibundus* sowie dem Populationstyp beeinflusst wird. Während sich die meisten gemischt-ploiden Populationen aus Mitteleuropa und dem östlichen Mitteleuropa genetisch nicht sehr unterscheiden, zeigen einige Populationen aus der Ukraine ein deutlich anderes genetisches Profil. Dieses Ergebnis wird durch den Fund ungewöhnlicher mitochondrialer DNA-Typen in Individuen jener Populationen bestätigt und legt die von den gemischt-ploiden Populationen Nord- und Mitteleuropas unabhängige

Entstehung jener östlichen Populationen nahe. In der Diskussion interpretiere ich diese Ergebnisse mit Bezug auf nach- und zwischeneiszeitliche Kolonisationsszenarien in Europa.

Kapitel zwei präsentiert eine Studie, welche in Zusammenarbeit mit Nicolas Pruvost durchgeführt wurde und in welcher wir Kreuzungsexperimente und Analysen von Mikrosatelliten-DNA benutzten, um fünf Populationen von unterschiedlicher Populationsstruktur zu vergleichen. Dafür untersuchten wir mit Hilfe von Indizes für Heterozygotie und genetische Differenzierung die Interaktionen zwischen verschiedenen Genotypen (LL, LLR, LR, LRR and RR). Die Ergebnisse dieser Studie erlaubten uns verschiedene Fortpflanzungssysteme zu definieren und zu unterscheiden, sowie ein evolutionäres Szenario für das Auftreten und die Aufrechterhaltung eines alternativen Systems gemischt-ploider Populationen in Mitteleuropa vorzuschlagen.

In **Kapitel drei** befasste ich mich mit den bioakustischen Eigenschaften männlicher Paarungsrufe innerhalb und zwischen gemischt-ploider sowie gemischten Populationen aus Hybriden und den Elternarten *P. lessonae* und *P. ridibundus*. Aus der Analyse von Feldaufnahmen der Rufe leitete ich fünf Rufparameter ab, welche alle einen Dosiseffekt des jeweiligen Genoms L oder R zeigten, d.h. sie nahmen mit steigendem L:R-Verhältnis der Genotypen in der Reihenfolge LL-LLR-LR-LRR-RR entweder zu oder ab. Zwei der fünf Rufparameter unterschieden sich zudem zwischen Populationssystemen. Die Effektgrössen nahmen in der Reihenfolge Genotyp-Populationssystem –geographische Lage der Population ab. Die Rufe diploider Hybriden (LR) variierten zwischen den Populationssystemen in Abhängigkeit davon, ob die Hybriden zur erfolgreichen Fortpflanzung eine der beiden Elternarten benötigen, oder nicht.

In **Kapitel vier** überprüfte ich innerhalb dreier Teiche (zwei mit gemischt-ploiden Populationen, einer mit einer Population aus LR-Hybriden und *P. lessonae*), ob sich das räumliche Mobilitätsmuster und Distanzverhalten der Männchen während der Paarungszeit zwischen Genotypen unterscheidet. Darüber hinaus testete ich die räumlichen Parameter auf Zusammenhänge mit der Körpergrösse und Kondition der Männchen, sowie mit der beobachteten Häufigkeit, in der die einzelnen Männchen im Amplexus mit Weibchen beobachtet wurden. Die Ergebnisse zeigten, dass weder Genotyp noch Kondition das räumliche Bewegungsmuster beeinflussen und deuteten darauf hin, dass vorhandene Unterschiede zwischen den Teichen wahrscheinlich auf

Unterschiede in der Populationsdichte zurückzuführen sind. Die Verteilung der Genotypen der Männchen im Amplexus entsprach für gemischt-ploide Populationen der tatsächlichen Verteilung der Genotypen der Männchen im Teich. Bei den Amplexus-Männchen der gemischten Hybrid-*P. lessonae*-Population waren *P. lessonae*-Männchen leicht überproportional vertreten.

In **Kapitel fünf** präsentiere ich eine kollaborative Studie mit Anke Stöhr über Ranavirus-Infektionen in wilden Wasserfrosch-Populationen. Die Studie kombiniert die Fallstudie eines Ranavirus-Ausbruchs unter in Gehegen gehaltenen Wasserfröschen mit der Beschreibung eines neuen Ranavirus und dessen phylogenetischer Klassifizierung.

Die Kapitel eins bis vier meiner Dissertation ermöglichen ein tieferes Verständnis der Diversität, Verbreitung sowie der genetischen und phänotypischen Differenzierung von *P. esculentus*-Populationen. Als Schlussfolgerung daraus argumentiere ich, dass es keinen „Allerwelts"-Hybriden gibt, sondern dass Teichfrosch-Populationen in Europa so divers sind, dass sie als signifikante Evolutionseinheiten denselben Respekt und dieselbe Aufmerksamkeit verdienen wie „reine" Arten.

General introduction

The evolutionary potential of hybridization

Hybridization has long been underestimated as an evolutionary mechanism, as it was seen as a rare event without evolutionary consequences (reviewed by Burke and Arnold 2001). Hybrid organisms were considered either unviable or less fit than their “good” species parents. In recent decades, however, more and more viable and successful hybrid systems have been detected, which shed a new light on the evolutionary consequences of hybridization. Especially the question whether hybridization could be another evolutionary pathway to speciation and thus a mechanism that generates diversity, has been subject to vivid discussion (Abbott 1992, Bullini 1994, Arnold 1997, Dowling and Secor 1997, Mallet 2007, Abbott et al. 2008). Most recent authors agree that the occurrence of hybridization events is not irrelevantly rare on the species level (depending, though, on the definition of the term). Indeed, it is estimated that the hybridization rate per species ranges between 1-10% in animals (Mallet 2005, Schwenk et al. 2008) and up to 25% in plants (Mallet 2005). Thus, a considerable number of plant and animal species are or have been involved in interspecific hybridization in some way or another.

Under favorable conditions, hybrid fitness can exceed the fitness of their parental species (Arnold and Martin 2010), which is why the formation and spread of hybrid taxa is not always seen as an ecological merit, but sometimes as a threat to the parental species if they are outcompeted by hybrid genotypes (e.g. Abbott 1992, Ayres et al. 2004). But in order to establish viable hybrid lineages that selection can act upon, hybrids have to overcome a series of challenges that make their way towards evolutionary success appear difficult. The challenges begin on the zygotic level, since difficulties in the meiotic pairing of chromosomes from genetically divergent parental species can either cause zygotic mortality of the hybrid offspring, or in case the offspring survive through embryonic development to adulthood, lower their fitness or render them infertile. On the zygotic level, some hybrid taxa evolved alternative reproductive mechanisms to overcome incompatibilities between parental genomes. Some successful vertebrate hybrids have adopted modes of asexual reproduction that render the necessity of recombination obsolete (Dawley 1989). These modes include 1) parthenogenesis, where females produce ovae with

unreduced chromosome numbers that develop without fertilization by males, 2) gynogenesis, where females produce unreduced gametes and development only requires the mere contact with sperm, but not the incorporation of paternal genetic material, and 3) hybridogenesis, where one of the parental genomes is discarded prior to meiosis and gametes contain only the remaining part of the genome, which was clonally transmitted without recombination. The mechanism of hybridogenesis was first described by Schultz (1969). Thus, by circumventing the challenges of normal meiosis and recombination, asexually reproducing hybrids both gain genetic diversity by combining two different parental genomes and benefit from the advantage of clonal reproduction (Bullini 1994).

An alternative way to overcome genetic incompatibilities is polyploidization (Dufresne and Herbert 1994, Otto and Whitton 2000, Otto 2007): Hybridization between genetically divergent species can change the genomic architecture of the resulting hybrid organism (Schwenk et al. 2008) and increase the likelihood of polyploidization in natural hybrids (Chapman et al. 2007). Polyploid hybrids carry more than one complete set of chromosomes from one or both parental species, thus yielding a higher ploidy level than in the involved parental species (Vrijenhoeck 1989, Ramsey and Schemske 1998). Polyploidy is a consequence of the production of unreduced gametes and their successful fusion and development. Through genetic and epigenetic interactions between genes that are present in several copies in polyploid organisms, polyploidy has genetic and somatic consequences that can provide additional fitness advantages to hybrid organisms, e.g. through heterosis effects, masking of harmful recessive alleles or other changes in the genomic structure resulting from genome duplication (Comai 2005, Mable et al. 2011, Mable 2013). Among vertebrates, some fish and amphibian hybrid systems have evolved successful and stable polyploid lineages (Kawamura 1984, Ptacek et al. 1994, Roberts 1997, Alves et al. 2001, Martino and Sinsch 2002, Holloway et al. 2006, Stöck et al. 2006, Vrijenhoek 2006), often in combination with asexual reproduction (Schultz 1969, Sousa-Santos et al. 2007, Cunha et al. 2008, Stöck et al. 2011, Choleva et al. 2012).

Success in hybrids might not only be a result of fitness advantages in direct competition against the parental species, but in some cases could have been facilitated by favorable historical and geographic conditions. Indeed, hybridization is considered to play a role in adaptive radiation of populations that extend to and

colonize new environments where they encounter other populations (Seehausen 2004). Under such a scenario, hybridization could elevate the response to selection and might cause populations to rapidly diversify under disruptive or divergent selection. In Europe, glacial and inter-glacial periods that caused populations to retreat to favorable Southern climates during cold periods and expand again northwards when the climate grew warmer, were important drivers of speciation and diversification (Hewitt 1996, 1999, 2004, Gante et al. 2009) and created hybrid zones between species (Hewitt 2001). Within these hybrid zones, two or more species that were formerly geographically isolated now co-exist again in sympatry again after post-glacial expansion and regularly hybridize. Depending on the viability and performance of the resulting hybrids, these can have a major impact on the parental species they originated from (Schwenk et al. 2008, Lehtonen et al. 2013).

The Pelophylax esculentus complex

The model organism of the present thesis is a versatile example of a successful hybrid system which adapted an asexual mode of reproduction (hybridogenesis) and has a potentially major impact on the existence of its parental species through a) clonally reproducing lineages excluding the genome acquired through backcrossing with the parental species in a form of sexual parasitism, and b) allopolyploid lineages reproducing independently from the parental species and therefore being able to extend their distribution within and outside the parental species' range. This model organism is the edible frog *Pelophylax esculentus* (formerly *Rana esculenta*), which was originally described as a true species by Carl von Linné (Linnaeus 1758) but is a natural hybrid between the two European water frogs species *P. lessonae* (genotype LL) and *P. ridibundus (*genotype RR) (Berger 1967). In general, diploid *P. esculentus* hybrids (genotype LR) form widely distributed populations in sympatry with one or both of the parental species which the hybrid needs for successful reproduction, due to its hybridogenetic reproduction (Tunner and Heppich-Tunner 1991, Morozov-Leonov et al. 2009, Zalesna et al. 2011). Most common are the L-E system (*lessonae-esculentus*), where P. *esculentus* interbreeds with *P. lessonae* to regain the previously eliminated L genome and the R-E system (*ridibundus-esculentus*), where the hybrid backcrosses with *P. ridibundus* to regain the eliminated R genome. In both systems, *P. esculentus* functions as a sexual parasite and the parental species are sexual hosts (Graf and Polls Pelaz 1989). Molecular studies indicate that

there were repeated events of primary hybridization (Spolsky and Uzzell 1986, Graf and Polls Pelaz 1989, Guex et al. 2002) and clonal gametogenesis was probably introduced into the hybrid system by *P. ridibundus*, which varies geographically in this ability (Hotz et al. 1985).

Several authors have described derivates from the L-E and R-E system which differ by their genotype and gender composition, and also by the occurrence of polyploid individuals (Graf and Polls Pelaz 1989, Plötner 2005). The so-called E-E (*esculentus-esculentus*) system includes both diploid and polyploid *P. esculentus* genotypes. Parental genotypes are usually absent from these all-hybrid populations, but this does not pose problems on the hybrids' reproduction, because accumulation of deleterious mutations through Muller's ratchet (Muller 1964) is prevented through recombination in polyploid individuals (Günther 1970, 1975, Christiansen and Reyer 2009). This is due to "meiotic hybridogenesis" (Alves et al. 2001), a mechanism derived from the usual genome exclusion in diploid individuals: triploid individuals exclude the genome present in a single copy (Günther et al. 1979, Vinogradov et al. 1990), recombine the genome that is present in a double copy and transfer these two copies into haploid gametes (Morishima et al. 2008, Christiansen and Reyer 2009). Recent studies found that these recombining polyploids are mostly triploids and -only rarely- tetraploid individuals (Arioli 2007, Jakob 2007, Christiansen 2009). Very recently, even a viable pentaploid individual has been identified (Hermaniuk et al. 2013). While tetra- or pentaploids are very rare in natural populations and their reproductive potential and gamete production patterns vastly unknown, the two most common triploid forms (LLR and LLR) are widespread in several parts of Europe, and their reproductive patterns have been studied intensely within the last few decades (Günther 1970, 1975, Günther et al. 1979, Ebendal and Uzzell 1982, Günther 1983, Günther and Plötner 1990, Plötner and Klinkhardt 1992, Tunner and Heppich-Tunner 1992, Brychta and Tunner 1994, Tunner 1994, 2000, Vorburger 2001, Christiansen et al. 2005, Rondinelli 2006, Som and Reyer 2006, Arioli 2007, Jakob 2007, Christiansen 2009, Christiansen and Reyer 2009, Arioli et al. 2010, Jakob et al. 2010, Hermaniuk et al. 2013, Pruvost 2013). Within the parental species' distribution ranges, diploid-triploid *P. esculentus* populations (hereafter called mixed-ploidy systems) can co-occur and interbreed with parental genotypes.

Approach to my study questions

Hybrid systems provide a great opportunity to study the interactions between genetic and ecological differentiation (Schwenk et al. 2008). In the case of *P. esculentus*, this hybrid complex offers indeed a complex array of phylogeographic, genetic and ecological questions involving different reproductive systems ranging from obligatory sexual parasitism in hybrid-parental populations to reproductive independence in mixed-ploidy populations. When studying such a complex system, changing the view from a large to a small scale can be both challenging and enlightening. In the following chapters, I tried to draw a line from the phylogeographic distribution of different *P. esculentus* population systems across a large part of the European continent (with a special emphasis on population systems involving polyploids) to the phenotypic implications polyploidy might (or might not) have on certain genotypes among several polyploid populations and within individual ponds.
Although my focus was clearly on mixed-ploidy systems, I also included a comparison with diploid hybrid-parental systems in my study questions, since I expected that the difference between asexual reproduction and reproductive modes involving recombination could potentially bear genetic and phenotypic consequences that should not be neglected.

Due to their patchy distribution, mixed-ploidy *P. esculentus* populations have been mostly studied on a regional scale or within country borders. Thus, in order to compare mixed-ploidy systems from different regions, it was necessary to sample frogs across a larger geographic area. Most records of mixed-ploidy *P. esculentus* populations exist from Scandinavia (Ebendal 1979, Fog 1994, Rybacki 1994, Christiansen et al. 2005, Arioli et al. 2010, Christiansen and Reyer 2011), Central and East-Central Europe (Günther 1970, 1975, 1983, Berger 1988, Blommers-Schlösser 1990, Günther 1990, Günther and Plötner 1990, Plötner 1990, Plötner and Klinkhardt 1992, Tunner and Heppich-Tunner 1992, Berger and Berger 1994, Plötner et al. 1994, Mikulicek and Kotlík 2001, Rybacki and Berger 2001, Plötner 2005) and Eastern Europe (Borkin et al. 2002, Borkin et al. 2004, Borkin et al. 2006).

My objective in **chapter 1** was to cover these three areas in a comprehensive geographic and population genetic study. I did this in collaboration with my PhD colleague Nicolas Pruvost. The focus of this study lay on the distribution of diploid and mixed-ploidy *P. esculentus* hybrid populations across a large part of Europe and on a genealogical comparison of populations using different molecular genetic

methods. Most samples were collected by Nicolas Pruvost and myself on extensive field trips. Additional samples were kindly provided by colleague scientist from various countries.

In **chapter 2**, Nicolas Pruvost and I performed crossing experiments on frogs from some of the populations sampled in chapter 1 to study gamete productions patterns among diploid and mixed-ploidy populations from different areas and assign these patterns to population genetic indices. This approach yielded the identification of three breeding systems and the proposition of an evolutionary scenario for the origin and maintenance of these systems.

Switching the focus to phenotypic characteristics potentially influcenced by polyploidy, **chapter 3** deals with bioacoustic differentiation of advertisement calls by water frog males within and between different breeding systems. My special interest was on the question whether the specific genome composition of a male from a mixed-ploidy population affects its call characteristics and makes it distinguishable from males of different genome compositions from the same pond. Furthermore, I answered the question whether there are bioacoustic differences between hybrids from diploid and mixed-ploidy population systems.

In **chapter 4**, I extended the question whether differences in genome composition in mixed-ploidy population can result in phenotypic differentiation to the spatial behavior of males during breeding season. For this I compared spatial movement parameters and inter-male distance of males between three ponds. Two of these ponds belonged to a mixed-ploidy system and were sampled by myself. To compare these data to a pond belonging to a diploid parental system, I was able to use spatial samples which were collected by Gaby Abt Tietje during her PhD work more than twenty years ago.

Chapter 5 presents an additional publication that is not directly related to the other chapters. The idea to this chapter was born out of the necessity to find the cause of a severe disease outbreak among water frogs in captivity during my first PhD year. Although tragic at first, this disease outbreak resulted in a fruitful collaboration with a veterinary PhD student (Anke Stöhr) from Germany which led to one of the first publications on ranavirus-infected amphibians from wild populations, the detection of a new ranavirus that could be traced back to its locality of origin, and to recommendations for reducing infection risks when keeping wild-caught amphibians in captivity.

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Genetic diversity and distribution patterns of diploid and polyploid water frogs (*Pelophylax esculentus*) across a large area of Europe

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Abstract

Allopolyploid hybridization is a rare, yet sometimes successful way in animals and plants to increase diversity by creating new geno- and phenotypes that manage to extend into new habitats or to adapt to environmental changes. The hybrid water frog *Pelophylax esculentus* resulted from hybridization between two distinct water frog species (*P. lessonae* and *P. ridibundus*) which probably crossed repeatedly during interglacial periods of the Pleistocene. Today *P. esculentus* is widespread in Europe and occurs in exclusively diploid mixed populations with one of the parental species, but also in populations containing diploid and polyploid hybrids, with or without one of the parental species. The distribution of these polyploid populations is patchy. This study investigates variation in genetic diversity between water frog populations with polyploid and/or diploid hybrids in respect to geographic location and the presence of the two parental species. Our results show that genetic diversity based on microsatellites is structured by geographic latitude and longitude, the presence of parental genotypes and the population type as defined by genotype composition. Analyses of microsatellite profiles identified two major genetic clusters and yielded similar results for the two parental genomes, except for a few areas where L- and R- clusters were not congruent between different clustering methods. Most polyploid populations from Central and East-Central Europe did

not genetically differ substantially, but the easternmost populations from Ukraine showed a distinctively different genetic profile, which was also confirmed by the novel finding of Anatolian water frog mtDNA in a *diploid P. esculentus* and the presence of *ridibundus*-specific mtDNA in polyploid *P. esculentus* specimen. Our findings suggest a different phylogenetic origin of polyploid water frogs from this area. We discuss these results with regard to possible hybridization and postglacial re-colonization scenarios in Europe.

Introduction

Natural hybridization and genetic diversity

In an ever changing environment, natural selection is the force that shapes the diversity of life forms, and genetic diversity is the raw material selection can act upon. There are many evolutionary mechanisms that create or reduce variability in genomes, most of them through an interaction of genetic and environmental processes. While some processes result in genetic drift or loss of genetic diversity as a consequence of population decline (Amos and Balmford 2001), others can increase genetic diversity and offer populations the potential to use new resources, expand into new habitats and evolve into new species.

Interspecific hybridization is amechanism that has long been considered evolutionary unimportant and maladaptive, but with new insights and a change of view on traditional species concepts, hybridization has been rehabilitated as another pathway that can increase genetic diversity and even lead to speciation (Arnold 1997, Mallet 2007). Many contemporary plants and animals show genetic evidence of past hybridization and introgression events (Arnold 1997, Dowling and Secor 1997), and some of these events resulted in stable hybrid taxa that have persisted over long periods of time. Interspecific hybrid taxa have often evolved genetic or genomic adaptations to circumvent meiotic disturbances during gametogenesis of heterozygote genomes, e.g. clonal reproduction in parthenogenetic, gynogenetic or hybridogenetic organisms, or through the production of diploid gametes and allpolyploid offspring. Allopolyploid hybrids have long been considered to be extremely rare in animals (Mable 2004), but over the past decades more and more independent allopolyploid taxa have been found to exist in vertebrates, i.e. in several genera of fish and amphibians (Gerhardt et al. 1994, Haddad et al. 1994, Ptacek et al. 1994, Becak and Becak 1998, Alves et al. 2001, Martino and Sinsch 2002, Holloway et al. 2006, Vrijenhoek 2006, Choleva et al. 2008). Polyploidy can thus be considered another stepping stone towards speciation, and although it might not be the most

common way among vertebrate taxa, for some it might be just the right way at the right evolutionary time.

Patterns of genetic diversity across Europe

In Europe, one evolutionary significant time was the series of ice ages and interglacials of the late Pleistocene (130'000 – 10'000 years ago). This period had a strong impact on the diversity and distribution of species we find today (Taberlet et al. 1998). During strong climatic oscillations, many species moved their distribution range between higher and lower latitudes and retreated to smaller refugia in the South or Southeast of Europe during glacial periods, where genetic sister lineages could evolve and hybridize again during later expansion (Hewitt 1999, 2011). Since recolonization was mostly a repetitive process, with fast northward expansion from the refugia during warm periods and subsequent contractions of ranges during cold periods (Hewitt 1996), successive genetic bottlenecks and loss of genetic diversity are probably the reasons why we observe a decrease of genetic diversity from south to north in many European species (Hewitt 1999). Additional to a latitudinal genetic diversity cline, some continental species that used eastern refugia show a longitudinal genetic diversity gradient from a more diverse East towards a genetically poorer West (Schmitt 2009). These recolonization patterns have furthermore created contact or 'suture' zones (i.e. a geographical band of range overlap) between species that have genetically diverged during glacial periods and hybridized again during postglacial expansion (Remington 1968, Taberlet et al. 1998).

Distribution and ecology of P. esculentus and its parental species

For our investigation we chose a widely distributed European amphibian taxon, the edible frog *Pelophylax esculentus,* which unites hybrid origin, allopolyploidy, geographic and genetic variety, and both hybridogenetic and sexual reproduction. *P. esculentus* is a natural hybrid of two European water frog species, the pool frog *P. lessonae*, and the marsh (or lake) frog, *P. ridibundus*. The two parental species *P. lessonae* and *P. ridibundus* can be considered true

continental species, with distributions extending from France as far as Russia (*P. lessonae*) and from the Rhine valley far into the Caspian Sea area (*P. ridibundus*) (Plötner 2005). Both species' distributions do not extend into Northern Europe, although a small and isolated metapopulation of *P. lessonae* exists in Sweden (Sjögren 1991), and some *P. ridibundus* populations occur at higher latitudes in the Baltic States. While *P. ridibundus* is widely distributed in areas around the Eastern Mediterranean Sea and the Black Sea, *P. lessonae* meets its southern distribution boundaries in Italy, where it overlaps in a contact zone with its sister species, the Italian pool frog *P. bergeri* (Plötner 2005). Molecular evidence indicates that Italy was the main glacial refugium from where *P. lessonae* subsequently recolonized northwards, probably following a colonization route bifurcating westwards and northwards after the passage of the Alpine-Carpathian gap (Zeisset and Beebee 2001, Snell et al. 2005). *P. ridibundus* probably expanded from a refugium in the Balkan (Pagano et al. 2001). Hybridization between the two species possibly occurred repeatedly before the Pleistocene period and during Pleistocene interglacials (Uzzell 1982).

The edible frog *P. esculentus* is not a "normal" hybrid in the sense of traditional species concepts that considered interspecific hybrids evolutionary dead ends (reviewed in Dubois 2011). In fact, it is one of the most common and wide-spread amphibian taxa in Europe. Its distribution range overlaps with or even extends those of its two parental species (Plötner 2005). In wide parts of its distribution, *P. esculentus* occurs only in sympatry with one of its parental species. Because of a reproductive mode called hybridogenesis (Schultz 1969), hybrids exclude one of their heterospecific chromosome sets during gametogenesis (either the "R" set inherited from *P. ridibundus*, or the "L" set from *P. lessonae*), while the retained genome is passed on clonally By back-crossing with the sympatric parental species (which carries the genome part excluded by the hybrid during gametogenesis) the excluded parental genome is regained to produce a new generation of heterospecific hybrids. These are hemiclonal individuals (Dawley 1989) that are usually unable to successfully procreate by mating with other hybrids, because of the irreversible accumulation of deleterious

mutations in the clonally transmitted genome (Vorburger 2001, Guex et al. 2002, Vorburger et al. 2009). In general, hybridogenetic *P. esculentus* are reproductively dependent on their syntopic parental species in many populations and therefore, are considered a sexual parasite (Graf and Polls Pelaz 1989) that need *P. lessonae* (L-E-population system) or *P. ridibundus* (R-E-population system) as sexual hosts.

In some areas, hybrid populations (*P. esculentus*) with allopolyploid individuals exist and can reproduce and persist independently of the parental species. These populations usually consist of both diploid (LR) and triploid (LLR and/or LRR) individuals and can occur either sympatrically with the two parental species, or without them in all-hybrid mixed-ploidy populations of the E-E (*esculentus-esculentus*) system (Graf and Polls Pelaz 1989). Beyond the distribution ranges of the parental species in Northwestern Europe, all-hybrid *P. esculentus* populations are the only native water frog populations, except for a small and isolated metapopulation of *P. lessonae* (Sjögren 1991). Here, triploid individuals usually produce haploid gametes, while diploid females produce both haploid and diploid gametes (reviewed by Günther 1983, Plötner 2005, later studies by Christiansen 2005, Arioli 2007, Christiansen 2009, Christiansen and Reyer 2009). The system is maintained by alternating between diploid individuals producing diploid gametes which fuse with haploid gametes into triploid offspring, and triploid frogs producing haploid gametes that fuse with other haploid gametes into diploid individuals (Som and Reyer 2006). When two diploid gametes fuse, viable tetraploid individuals (type LLRR) can occur, but in nature they are very rare and do not seem to have a fitness advantage over diploid or triploid individuals (Arioli 2007, Jakob 2007, Christiansen 2009, Arioli et al. 2010, Jakob et al. 2010). From all-hybrid populations in Denmark and Sweden we know that triploid hybrids (genotype LLR and LRR) can recombine their double-copy genome (Arioli 2007, Christiansen and Reyer 2009), thus providing a mechanism of maintaining genetic diversity and circumventing the danger of accumulating deleterious mutations in these populations.

The patchy distribution of polyploidy in P. esculentus

Despite the wide distribution of *P. esculentus* across Europe, the known distribution of mixed-ploidy populations is rather patchy, with areas in between where only diploid hybrids and parental populations are found. Polyploid forms (i.e. normally triploids of the type LLR or LRR) have been found and studied in the following countries: Denmark (Fog 1994, Christiansen et al. 2005), Sweden (Ebendal 1979, Ebendal and Uzzell 1982, Arioli et al. 2010, Jakob et al. 2010), Baltic States, Germany (e.g. Günther 1970, 1975, Günther and Plötner 1990, Plötner and Klinkhardt 1992, Berger and Berger 1994, Rybacki 1994), Poland (e.g. Berger 1988, Rybacki and Berger 2001, Czarniewska et al. 2011), Austria (Tunner and Heppich-Tunner 1991, Tunner 1994, Czarniewska et al. 2011), Czech Republic (Pruvost 2013), Slovakia (Mikulicek and Kotlík 2001, Pruvost et al. 2013), Hungary (Tunner and Heppich-Tunner 1992, Brychta and Tunner 1994), Ukraine (Borkin et al. 2004, Mezhzherin et al. 2010) and Russia (Borkin et al. 2006). Depending on the geographic area, polyploids are formed in two principally different ways

1. In most regions, triploid individuals of two types (LLR and LRR) and both sexes arise from the fusion of haploid L or R gametes (produced by triploid hybrids of the LLR and LRR type, but also by diploid LR hybrids) and diploid, heterospecific LR gametes which are produced in varying proportions by LR hybrids (Christiansen et al. 2005, Arioli 2007, Christiansen 2009, Christiansen and Reyer 2009).
2. In a few areas, triploid, exclusively male hybrids of type LLR are formed through the fusion of haploid R gametes (provided by female diploid LR hybrids) and diploid LL sperm (produced by triploid LLR males) (Tunner and Heppich-Tunner 1992, Brychta and Tunner 1994, Tunner 2000, Mikulicek and Kotlík 2001, Pruvost et al. 2013).

Objectives of this study

In Scandinavia, where mixed-ploidy all-hybrid populations are almost the only form of water frog populations, earlier studies have shown that the type of polyploidy (LLR, LRR) and their relative frequencies is determined by the types of gametes being produced (Christiansen 2009) as well as by environmental and geographic factors (Arioli et al. 2010, Jakob et al. 2010, Christiansen and Reyer 2011). However, since polyploidy in *P. esculentus* is not restricted to northern regions (see above), we aimed to investigate the population genetic patterns within and among polyploid *P. esculentus* populations across a larger European area. The objectives of our study were thus:

1. To examine **genetic diversity and genetic differentiation in water frogs over a large geographic scale and across a diverse selection of population types**. According to the post-glacial colonization theory we were especially interested whether populations differed in genetic diversity across areas along latitudinal and longitudinal clines. Alternatively, genetic diversity could be maintained by occasional crosses with parental species in populations where *P. esculentus* lives syntopically with the parental species. In this case, we would expect genetic diversity to be correlated with the presence of parental genotypes in the population.
2. To find **genetic structuring among polyploid populations** from different areas and genotypic composition. For this we used combined genetic information from microsatellites and mtDNA.

Methods

Sampling of genetic material

Water frogs were captured by hand in the field by the authors and by the colleagues listed in the acknowledgements. Tissue samples were taken from toe clips and stored in 70% ethanol until processing at the University of Zurich. Genotype and ploidy of individuals were determined via microsatellite analysis. DNA extraction, PCR and electrophoresis followed protocols as in Christiansen and Reyer (2009). We obtained genetic samples of water frogs from 72 populations in 15 countries (see Appendix 1 for a list and further details). In the following text numbers in square brackets refer to the populations listed in Appendix 1: e.g. [1] stands for the population in Uppsala. Short geographic distances (< 3km) between populations were the exception, as samples from nearby ponds with similar genotype composition and pond features were usually pooled and considered belonging to the same population.

We classified a population as of "mixed ploidy" when in addition to diploid hybrids at least a few polyploid hybrid individuals were found in the sample. Additionally, records from the literature or from personal communication by researchers familiar with the area confirmed the occurrence of polyploids for all populations where we found them in our study. Populations were classified as "diploid" when only diploid hybrids were found in the sample and when the absence of polyploid forms in this area was not contradicted by local colleagues or by the recent literature. Populations were further assigned to a population type based on cumulative information on the presence or absence of parental and diploid and polyploid genotypes in the population sample (Appendix 1). We distinguished between: all LL (only LL, no hybrids), all RR (only RR, no hybrids), diploid L-E (LL and LR), diploid R-E (RR and LR), diploid L-E-R (LL, RR and LR) and mixed-ploidy (any combination with polyploid genotypes).

Microsatellite marker selection and genotype determination

Microsatellite analysis was performed on samples from all 72 localities. To obtain useful data for subsequent population genetic analyses, it was important to select

a set of microsatellites that work on the broad geographical range of populations listed in Appendix 1, We therefore started out with a total of 18 microsatellites (Table 1) developed on water frogs (Garner et al. 2000, Zeisset et al. 2000, Hotz et al. 2001, Christiansen and Reyer 2009, Arioli et al. 2010). These markers were developed on and work well for Western and North/Central European populations, but allele distribution and variability in other European parts are not yet entirely known.

Table 1: Overview of the microsatellite markers tested and selected in this study. Allele numbers and genetic diversity for L (HeL) and R genomes (HeR) are given pooled across all samples. Four markers were not used in this study (explanation is given in the text).

Marker name	Author / GenBank ID	Allele numbers and genetic diversity in this study				
		L alleles	R alleles	both L + R	HeL	HeR
RICA1b6	Arioli et al. 2010 / EF121548	3	18	2	0.486	0.730
RICA1b5	Garner et al. 2000 / AF286388	7	8	0	0.092	0.275
Ga1a19 redesigned	Arioli et al. 2010 / EF121547	4	29	1	0.038	0.652
Res16	Zeisset et al. 2000 / AF195843	2	9	4	0.086	0.468
Rrid064A	Christiansen and Reyer 2009 / EU445524		19			0.733
Re2Caga3	Arioli et al. 2010 / EF121550		41			0.900
Res22	Zeisset et al. 2000 / AF195846		27			0.644
Rrid013A	Hotz et al. 2001 / FJ024047		6			0.249
Rrid059A redesigned	Christiansen and Reyer 2009 / FJ024048		29			0.645
Rrid135A	Christiansen and Reyer 2009 / EU445526		27			0.718
RICA2a34	Christiansen and Reyer 2009 / EU445521	18			0.734	
Ga1a23	Christiansen and Reyer 2009 / EU445523	22			0.860	
Ca1A27	Christiansen and Reyer 2009 / EU445522	15			0.827	
RICA18	Garner et al. 2000 / AF286386	22			0.687	
Re1CAGA10	Arioli et al. 2010 / EF121549	Not used in this study				
Res20	Zeisset et al. 2000 / AF195845					
Rrid169A	Christiansen and Reyer 2009 / EU445525					
RICA5	Garner et al. 2000 / AF286385					

The 18 primer pairs were combined in two multiplex mixes of 9 primer pairs each. Sometimes these multiplex mixes were split up into four mixes because allele overlap was observed during processing of samples from increasingly distant localities. Singleplex PCRs were run routinely to cross-check the results from individual primers and split-up primer mixes. We ran PCR products on an ABI

3730 Avant capillary sequencer (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) with an internal size standard (GeneScan-500 LIZ). Alleles were scored and peak heights were measured using program Genemapper 3.7 (Applied Biosystems 2004).

Multilocus genotypes were established from the allele data in a step-wise procedure. First, alleles were scored without knowledge of locality or genotype (LL, LLR, LR, LLRR, LRR, RR). Then, with a combination of field notes describing the supposed taxon of the individual based on morphological characters and prior expectations of L and R specificity from previous studies (Christiansen 2005, 2009, Christiansen and Reyer 2009, 2011), consensus genotypes were determined for all individuals. On the basis of these consensus genotypes, genome specificity was assigned to previously unknown alleles. The ploidy of the consensus genotypes was verified by analyses of dosage effects at four loci (Res16, Ga1a19, RlCA1b5 and RlCA1b6), following the method described in Christiansen (2005). Genotypes and peak sizes were manually proof-read, and plots of log10(height1/height2) were drawn for all pairwise combinations of alleles in the entire data set. These plots were visually examined for groups of individuals corresponding to 2:1, 1:1 and 1:2 allele ratios. Depending on the genome specificity of the alleles, these ratios could be translated into LL, LLR, LR, LRR, and RR genotypes (LLL, LLLR, LRRR and RRR were not found). Due to the low occurrence of LLRR in natural populations, the danger of misclassifying an LLRR individual as LR is very low (Christiansen 2009, Arioli et al. 2010). LLRR tetraploids might appear as LR at some dosage effect loci, since the allele ratio between L and R is 1:1. However, the chance of mistaking LLRR for LR is very low, since tetraploids are usually revealed by amplification of more than one L or R allele at one or the other locus when examined across a larger number of loci.

When we encountered alleles in an individual that were in conflict with the consensus genotype (e.g. a locus yielding two L alleles when LR was expected by consensus genotype), the sample were examined again or rerun in PCR and fragment analysis. Loci that were still incongruent (= alleles considered as R-

specific were found in L genomes and vice versa) with the consensus genotype after this extra round of evaluation were treated in one of three ways. When only few frogs from the same locality (populations with n < 15: 1-2 individuals; populations with n > 15: up to 3 individuals) showed the same kind of incongruence at the same locus, this locus was coded as missing data, as the problem was not quantitatively important. When more frogs were concerned, the problem could usually be assigned to either allele inspecificity (= the allele could not be assigned to either L or R because it repeatedly occurred in both) or null alleles (= giving no signal in the PCR because of mutations in the primer binding sites or complete absence of the microsatellite in the genome). In cases of allele inspecificity, single alleles were re-assigned to either the L or R genome to fit the consensus genotype. Some loci turned out problematic for one or both genomes (L: RlCA5, Res20, Rrid013A; R: RlCA2a34, Rrid169A; both L and R: ReCaga10), since missing values accumulated in high numbers and in a non-random pattern despite multiple re-runs of the affected samples; this indicated a systematic failure of sample batches rather than the occurrence of true null alleles.

Especially populations from Ukraine and Romania were affected by systematic failure of amplification in the R genome. We know from other genes that *P. ridibundus* is genetically very heterogeneous in some areas. In the Czech Republic, for example, comprising alleles are characteristic of Anatolian frogs (*P. cf. bedriagae*) and *P. kurtmuelleri* (Plötner unpublished). Therefore we suspected that the non-amplification in some of our populations from Eastern Europe might also be due to an admixture of genes from outside the Central European range of *P. ridibundus*. Existing values in the affected loci were thus only used for confirmation of the consensus genotypes, but in any subsequent analyses the whole locus was omitted for all populations. Also, one marker (Rrid059A) turned out almost monomorphic in the L genome by yielding only two alleles across all samples. To avoid strong differences in polymorphy among markers, the affected locus was omitted from the L genome data set. Table 1 gives an overview of the 4 markers that were omitted from the analyses and the 14 markers that were

eventually used: 4 for the L genome, 6 for the R genome, and 4 that amplified in both genomes.

Null alleles can bias the estimates of population genetic parameters when they occur in high frequencies. In our study we did not often observe persistent non-amplification after repeated runs of samples.We thus assume that true null alleles occurred at low frequencies only, which was also found in an earlier study on water frogs by Christiansen (2009). We expected that null alleles should either be directly detectable in the four dosage effect loci (in the case of a null allele, the actual dosage ratio did not fit the ratio expected by the consensus genotype), or by non-amplification of non-dosage markers in individuals that have one copy of the genome and should show a peak in this locus. For example, a null allele occurring in the L genome at a non-dosage-specific locus would not be detectable in a LLR or LL frog that possesses two different alleles in this locus. But a null allele would be unmasked when both L genomes possess the null allele (thus none of them amplifying), and also in LR and LRR frogs that carry only one copy of the L genome (non-amplification in the single genome). Non-amplification was verified through repeated runs of the affected sample. We had two populations with persistent non-amplification, one in the L genome (57] and one in the R genome [20]. However, as only few individuals (4 samples out of 33, respectively 3 out of 29) were affected, the locus was coded as missing data in these individuals.

Microsatellite data analysis

For population genetic analyses, the L and R genomes were split into two independent data sets and analyzed separately. This was done because some populations differed in sample numbers between the two data sets, since many of the sampled populations consisted of a mix of hybrid and non-hybrid frogs (i.e. *P. lessonae* and *P. ridibundus* of genotypes LL and RR), and since homospecific genotypes can only occur in one of the two data sets). Due to the mix of haploid and diploid genomes in our full data set, we restricted analyses on genetic differentiation and structuring to allele-frequency-based methods rather than

methods based on observed heterozygosity. As a measurement for genetic diversity we used He (expected heterozygosity according to (Nei 1978)) calculated by program SPAGeDi 1.3 (Hardy and Vekemans 2002). SPAGeDi can handle a mix of haploid and diploid data and was further used to calculate Nei's D, Fst and geographic distance matrices among populations. To test the influence of population parameters in genetic diversity based on microsatellite data, we performed generalized linear models in program Systat 11 (SYSTAT Software Inc. 2004). Mantel tests between genetic distance and geographic distance matrices were calculated with program zt (Bonnet and Van de Peer 2002). Pairwise geographic distances were calculated from GPS coordinates using the online software Geographic Distance Matrix Generator version 1.2.3 (Ersts 2012). Cluster analyses for L and R genomes in mixed-ploidy populations were performed on the basis of pairwise Nei distances, using the Euclidian distance metric in the software PermutMatrix Version 1.9.3 (Caraux and Pinloche 2005). Since results from cluster analyses can vary with the cluster algorithm used, we compared trees resulting from three different linkage algorithms implemented in the PermutMatrix Version 1.9.3 software: single linkage (nearest neighbor), average linkage (UPGMA) and Ward criterion (Ward 1963).

Mitochondrial DNA sequence analysis

To estimate a haplotype genealogy across a large area, we analyzed mtDNA sequences of 1175 samples from 105 localities that were representative for the range of geographic and population type variation (Appendix 2). For three reasons, the number of localities and samples used for mtDNA sequence analysis derived from the number used for microsatellite analysis:

a) We did not perform mtDNA sequencing for all individuals of which we obtained microsatellite data (i.e. only subsamples of some populations were processed when low sequence diversity was expected based on preliminary analyses, or when several nearby populations of similar genotype and haplotype composition were available and thus rendered sequencing of all samples from this area redundant)

b) We added samples by including mtDNA sequences from an earlier study (Arioli 2007, chapter 5)

c) We included additional populations of which we had only very few samples and thus did not use them for microsatellite analysis.

DNA extraction, PCR and sequencing were conducted following closely the protocol described in Plötner et al. (2008). We sequenced two genes, ND2 (1038 bp) and ND3 (340 bp) in both directions using forward and reverse primers, which have previously been used and described in phylogenetic and phylogeographic studies (e.g. Plötner 2005, Arioli 2007, Plötner et al. 2008, Akin et al. 2010). Mitochondrial DNA sequences were initially aligned using the algorithm ClustalW implemented in program MEGA5.05 (Tamura et al. 2011). Subsequently, the alignment was improved manually. Using MEGA5.05, we performed sequence statistics and sequence model selection on the basis of hierarchical likelihood ratio tests, and estimated a haplotype genealogy based on 1378 concatenated ND2 and ND3 sequences using maximum likelihood as implemented in the software. Branch support was evaluated by bootstrapping (Felsenstein 1985) with 1,000 replicates.

Results

Population type and genotype distribution

Based on microsatellite profiles, we genotyped and analyzed a total of 2062 frog samples from 72 localities. The minimum distance between localities was 2.63 km, the maximum was 1863.5 km. Average geographic distance between populations was 727.8 km ± 427.7 km (1 S.D.). The most numerous taxon was *P. esculentus* with 63 % of all genotyped individuals, followed by *P. ridibundus* (25.5%) and *P. lessonae* (11.5%). *P. esculentus* occurred in 50 localities (69%), *P. ridibundus* in 40 (56%), and *P. lessonae* in 27 localities (38%). In our sample, we found all-*ridibundus* populations (n = 20, 26% of localities) almost exclusively south of 48° latitude and east of 16° longitude, especially in the proximity of the numerous tributaries to the Danube river and the Black Sea (Figure 1). One all-*ridibundus* population [7], however, was situated quite remotely from the rest in the Baltic area. We found only two all-*lessonae* populations ([1],[5], 2.6% of all samples), both of which were among the northernmost populations. The remaining populations included hybrid *P. esculentus*. Of these, 26 (36% of total) were classified as diploid, and 24 (33%) as mixed-ploidy populations that contained triploid LLR and/or LRR genotypes. All triploid individuals co-occurred with diploid LR hybrids, and in 50% of the mixed-ploidy populations polyploid hybrids additionally co-occurred with either *P. ridibundus* or *P. lessonae*. Only in two populations ([11] and [54]), diploid and polyploid hybrids occurred together with both parental species. Fourteen (17%) localities included both types of triploid hybrids, LLR and LRR. In another eight populations (11%), LLR was the only type of triploid hybrid, whereas five populations (7%) included only the LRR genotype. Only four tetraploids of the genotype LLRR were detected in three populations ([4], [11] and [24]), i.e. in 0.2% of all samples.

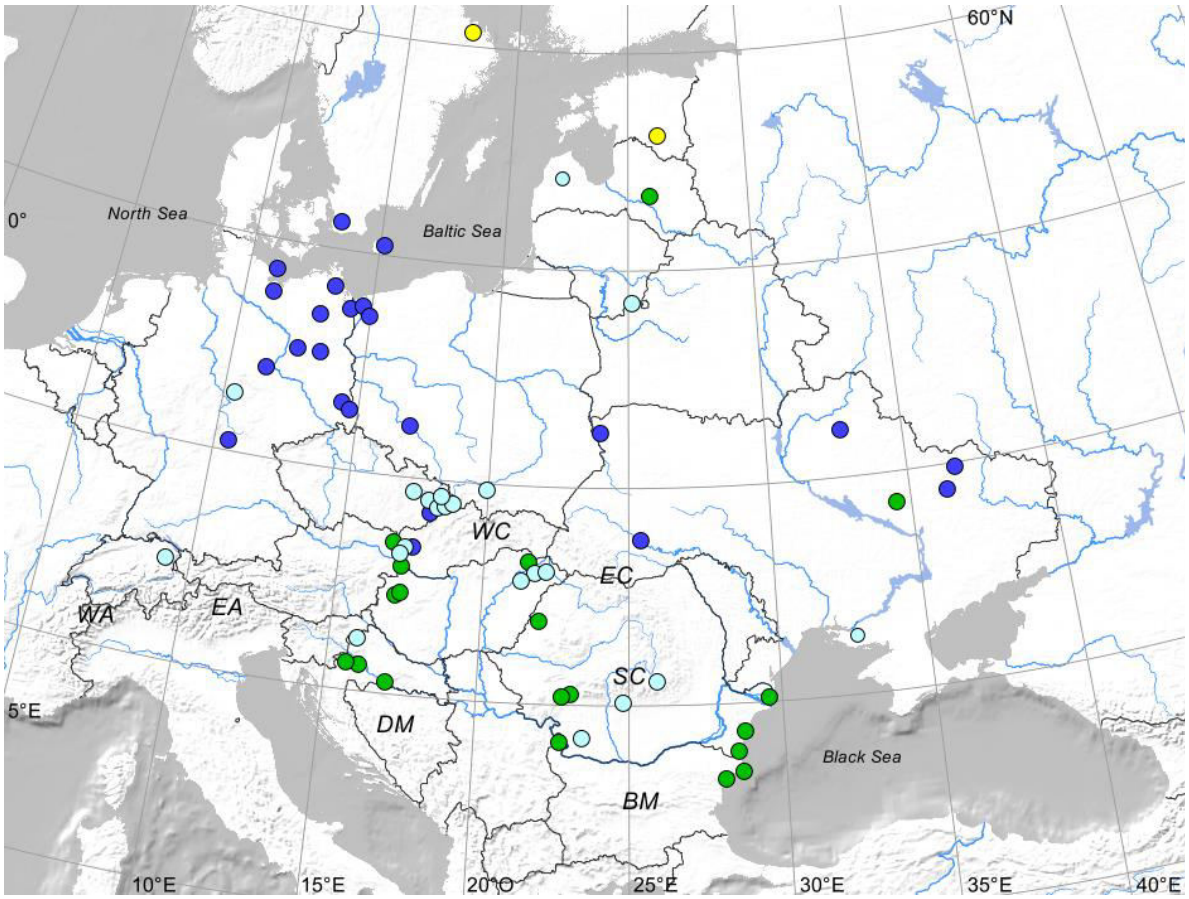

Figure 1: Sample locations for microsatellite analyses. Symbol colors indicate populations of the following types: yellow = pure lessonae, green= pure ridibundus, dark blue = mixed ploidy, turquoise = diploid hybrid with parental species *P. lessonae* or *P. ridibundus,* or both. Letters denote relevant mountain ranges, WA=Western Alps, EA=Eastern Alps, WC=Western Carpathians, EC =Eastern Carpathians, SC=Southern Carpathians, DM=Dinaric Mountains, BM=Balkan Mountains.

Effects of geographic and population parameters on genetic diversity

The loci used for analysis in the R genome (Table 1) yielded 220 alleles (range: 6-41 per locus) in total for a sample size of 1807 individuals from 66 populations. In the L genome, 100 alleles were found (range: 5-22 per locus) across the entire sample of 1506 individuals from 50 populations. Gene diversity was generally lower in the L genome than in the R genome (mean HeL: 0.321; mean HeR: 0.440). HeL was also lower than HeR in every marker amplifying both genomes (Table 1). The influence of geography on genetic diversity was evident in the three northernmost parental populations, where we found comparatively low values for genetic diversity: the remote all-*ridibundus* population [7] in the Baltic

area had the second lowest value for genetic diversity among all pure *P. ridibundus* populations (HeR = 0.48, average across all-ridibundus populations: HeR = 0.69), and genetic diversity in the two all-lessonae populations ([1]: HeL = 0.19, [5]: HeL = 0.28) was also below the overall average.

We investigated whether the specific geographic locality and population composition influences genetic diversity in the R and L genome. For HeL and HeR we performed separate GLMs in which we incorporated as independent variables geographic latitude, longitude, the proportion of parental (% LL, % RR) and polyploid genotypes (% polyploid), and also tested for interactions between geographic parameters and population type (Poptype x latitude, Poptype x longitude). Results are presented in Table 2. Both HeL and HeR show a negative relationship with increasing latitude, meaning that genetic diversity in both genomes decrease from lower to higher latitudes. Furthermore, higher percentages of parental genotypes had a positive effect on genetic diversity in both genomes in the model. In contrast, the effects of longitude (when referring to the whole data set) and percentage of polyploid genotypes were not significant to either HeL or HeR and therefore omitted from the model in a step-wise variable selection process. Interestingly, longitude did play a role for both HeR and HeL in the interaction with population type. Similarly, the model yielded a significant interaction between population type and latitude for HeL. This indicates that geographic parameters influence genetic diversity in only some population types. In Figures 2 and 3, these interactions between population type and geographic parameters are plotted and grouped by population type.

Table 2: Results from GLM analyses for HeL (genetic diversity in the L genome) and HeR (genetic diversity in the R genome) versus geographic and population parameters. Significant results are printed in bold. Values in brackets (-) are only shown for reference, since these variables were omitted from the model during step-wise variable selection.

	HeL				HeR			
Variables	df	coeff.	F	P	df	coeff.	F	P
Latitude	1	-0.026	23.30	**< 0.0001**	1	-0.020	19.05	**< 0.0001**
Longitude	(-)	(-0.092)	(0.36)	(0.552)	(-)	(0.009)	(0.04)	(0.945)
% LL*	1	0.001	4.43	**0.041**				
% RR **					1	0.002	5.29	**0.025**
% polyploid	(-)	(0.049)	(0.10)	(0.754)	(-)	(0.142)	(1.19)	(0.279)
Interactions								
Poptype x latitude	2	n.a.	5.14	**0.010**	2	n.a.	(0.30)	(0.878)
Poptype x longitude	2	n.a.	5.06	**0.011**	2	n.a.	7.17	**< 0.0001**
Error	43				59			

* only available in HeL data set **only available in HeR data set

The decrease in HeL with increasing latitude appears to be steeper in mixed-ploidy populations than in L-E populations (Figure 2a). However, the two population types are not distributed evenly across the latitudinal range. L-E populations are most numerous between 42°-50° latitude. Within this range, their genetic diversity in the L genome does not appear to decline. The rest of L-E populations ([2],[6],[8]) is situated between 54°-58° and averages lower in HeL than the group of L-E populations further south. Two all-lessonae populations ([1],[5]) that were also situated at high latitudes showed similarly low or even lower HeL than the three northernmost L-E populations. This could still indicate a regional decline of HeL at higher latitudes among populations with some proportion of LL, but since no data was available for the distribution gap between 50°-54°, one cannot tell whether the strong negative effect of higher latitude (as indicated by the GLM in Table 2) reflects a true cline among L-E populations. In contrast, such a cline is more obvious in the linear decline of HeL with increasing latitude among mixed-ploidy populations, which were fairly evenly distributed

over a range between 48°-56° latitude. This range overlapped only slightly with the distribution of L-E populations in our data (Figure 2b).

In terms of longitude (Figure 3), distributions of L-E and mixed-ploidy populations differed also, but in the almost opposite way. Polyploid populations were sampled mostly between 10°-20° longitude and only two populations ([49],[54]) bridged the gap between the west-central majority and the few polyploid populations ([50],[51],[52]) at > 32° longitude (Figure 2b). In contrast, L-E populations were most numerous in the range between 15°-26° longitude and were sampled only sporadically at lower [40] or higher [55] longitudes. While Figure 2b illustrates a slight decline in HeL with increasing longitude in L-E populations, a true linear relationship is not detectable for mixed-ploidy populations. Rather, HeL is variable at lower longitudes, but scores generally higher at longitudes > 25°. The other population types were too rare in our sample to detect general trends.

Concerning the R genome (Figure 3), genetic diversity HeR is generally lower in systems with hybrids than in pure RR populations. The lowest values of HeR are found in L-E populations. However, geographic parameters seem to play a role in genetic diversity only in pure RR and in polyploid populations but not in L-E populations. While HeR declines with latitude for both mixed-ploidy and pure RR populations, values for L-E populations are not linearly declining (Figure 3a). A difference between the three populations types is also visible in the plot of HeR against longitude, where we observe an increase of HeR with longitude in both all RR and mixed-ploidy populations, but no effect on L-E populations, where values of HeR appear to be even lower at high longitudes than at low ones (Figure 3b).

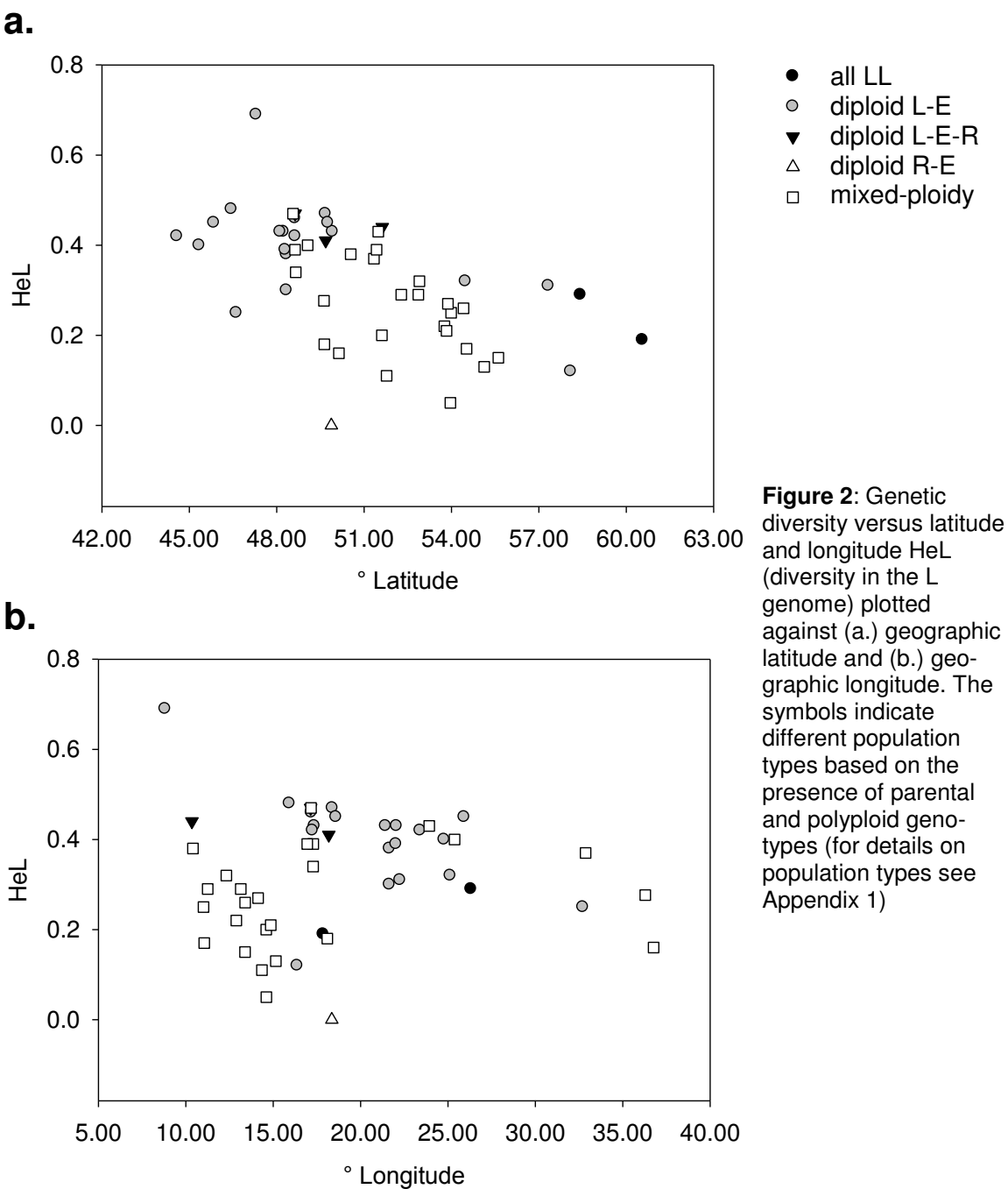

Figure 2: Genetic diversity versus latitude and longitude HeL (diversity in the L genome) plotted against (a.) geographic latitude and (b.) geographic longitude. The symbols indicate different population types based on the presence of parental and polyploid genotypes (for details on population types see Appendix 1)

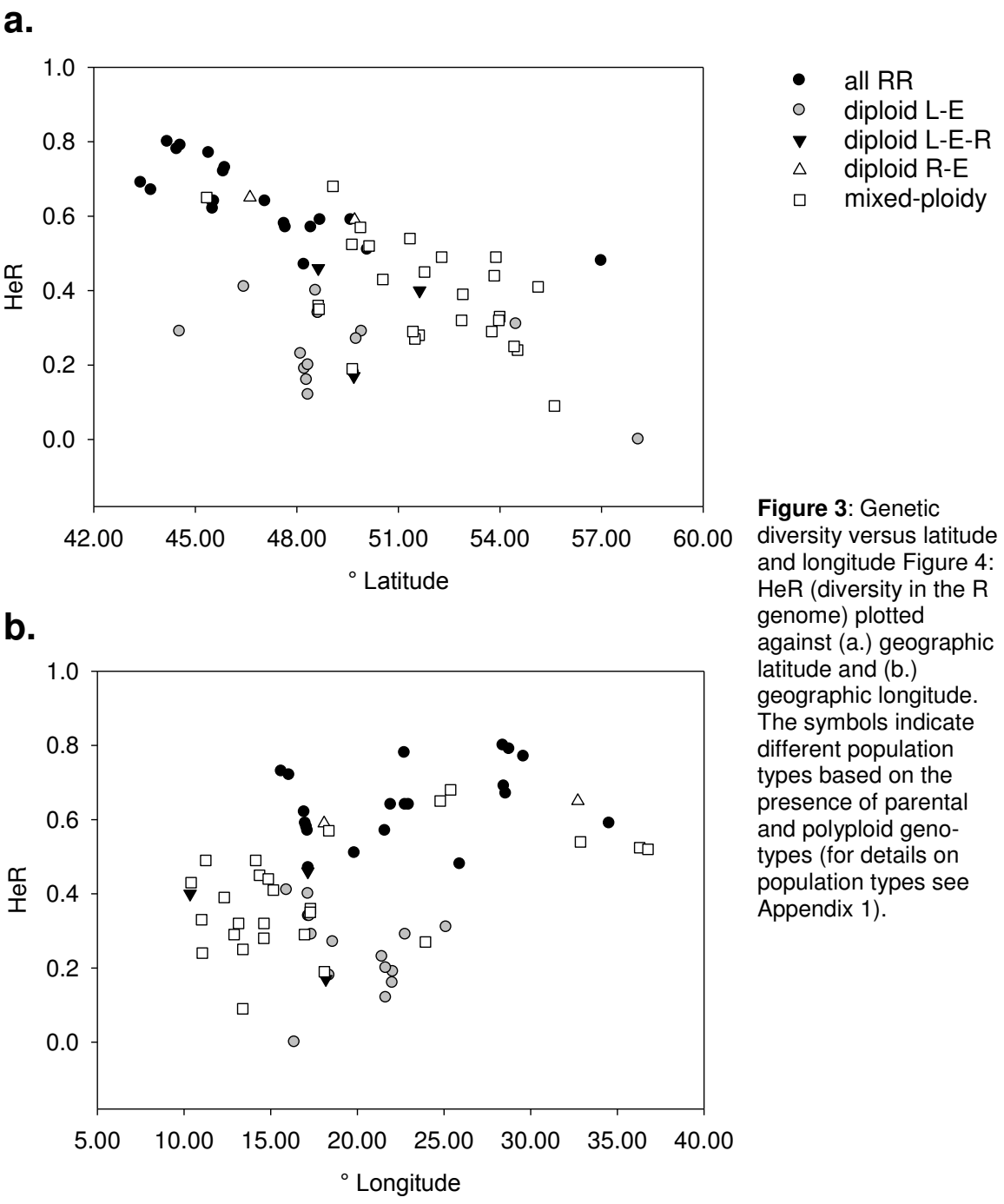

Figure 3: Genetic diversity versus latitude and longitude Figure 4: HeR (diversity in the R genome) plotted against (a.) geographic latitude and (b.) geographic longitude. The symbols indicate different population types based on the presence of parental and polyploid genotypes (for details on population types see Appendix 1).

Isolation by distance

For the entire sample, calculation of global Fst yielded 0.349 for the L genome and 0.294 for the R genome, thus attributing 34.9% of variation in the L genome and 29.4% of genetic variation in the R genome to inter-population differences. When we tested for isolation by distance across all populations, we found genetic distance (given as Nei's D_S) to increase strongly with geographic distance between populations in both genomes (one-tailed Mantel test: L: r = 0.63, p = 0.00001; R: r = 0.65, p = 0.00001; Figure 4). Mantel tests on Fst-values between populations yielded similar results, yet the effect was smaller (one-tailed Mantel test: L: r = 0.22, p = 0.00001; R: r = 0.35, p = 0.00001).

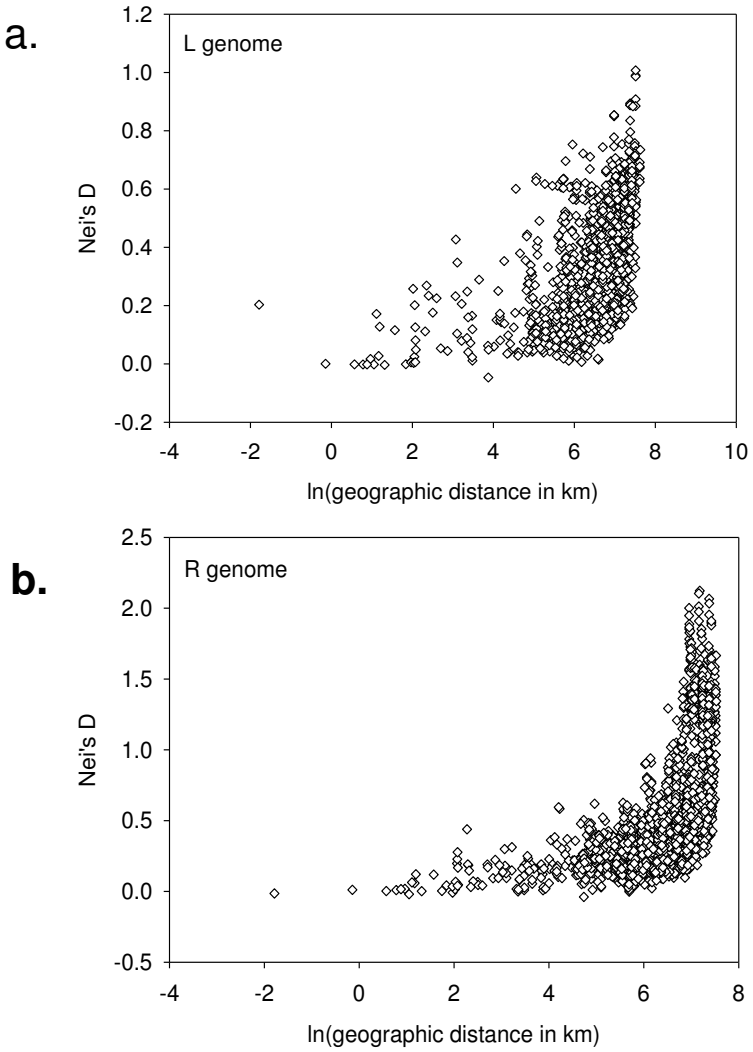

Figure 4: Isolation by distance in (a.) the L genome and (b.) the R genome. Pairwise distances between all 72 microsatellite samples are shown. Geographic distance was ln-transformed for better illustration.

Genetic structuring of mixed-ploidy populations

Isolation by distance was supported for the 24 mixed-ploidy populations in our data set (one-tailed Mantel test: L: r = 0.54, p = 0.0003; R: r = 0.43, p = 0.0035), showing that gene flow among these polyploid populations is similarly distance-dependent as in other water frog populations. In order to test for genetic structuring among polyploid populations, we performed separate cluster analyses for the L and R genome using three different linkage algorithms for the genetic distance (measured by Nei's D) between mixed-ploidy populations. Overall, these algorithms yielded similar statistical values, but created different cluster trees. The three cluster trees for the L genome (Figures 5a-c) have in common that two main clusters can be identified and that genetic differentiation within the larger cluster is lower (illustrated by dark colors) than within the smaller cluster (brighter colors indicate higher dissimilarity). One small but distinct cluster was formed by two populations [51] and [52], which was congruent across all three cluster methods. A second main cluster was formed by the rest of the populations. A subcluster of four populations ([33],[36],[50],[4]) showed distinct dissimilarity to the large main cluster and was assigned to either the main cluster (single linkage and average linkage) or to the small main cluster (Ward criterion). Within the large main cluster, substructuring was not congruent among trees and therefore not considered reliable. Cluster analyses for genetic distance in the R genome (Figures 6a-c) provided a similar picture: Two main clusters, one formed by the populations [51] and [52], the other consisting of the rest of populations. Within this large cluster one small, consistent subcluster of populations [50] and [54] could be distinguished in all three cluster trees. Clustering among the rest of populations, like in the L genome analysis, did not yield any consistent results. The geographic distribution of the main clusters and consistent subcluster from Figure 5a-c are illustrated in Figure 7a, the main clusters and distinct subcluster in the R genome (Figure 6a-c) are shown in Figure 7b.

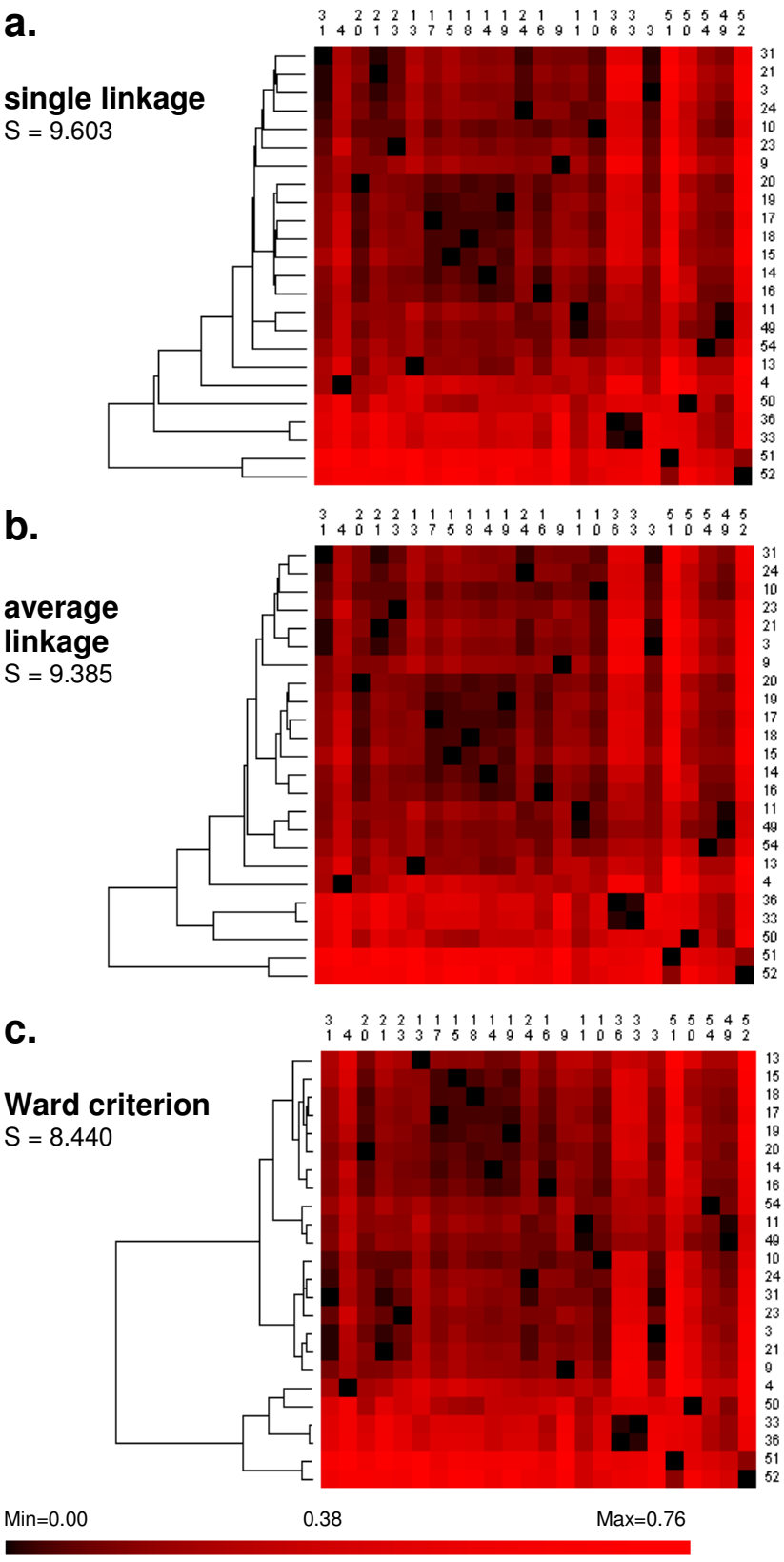

Figure 5: Cluster analysis of the L genome between all polyploid populations based on Euclidean distances of Nei's D and using (a.) single linkage, (b.) average linkage and (c.) Ward criterion clustering algorithm. The S (= path length) value stands for the sum of all pairwise distances between row neighbors. Population numbers given on the right and top correspond to numbers in Table Appendix 1. The darker the color, the lower the genetic differentiation between two populations.

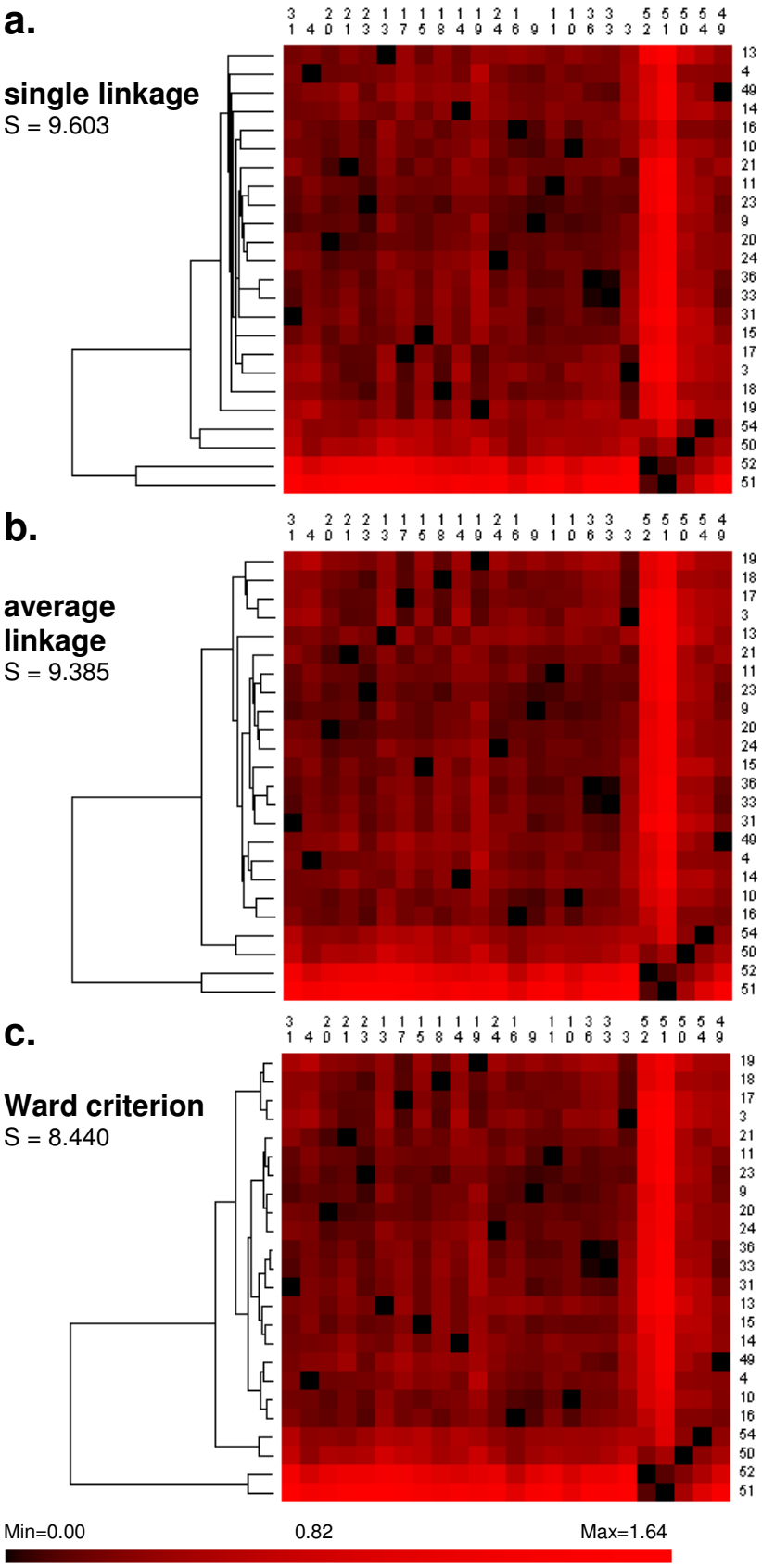

Figure 6: Clustering analysis of the R genome between all polyploid populations based on Euclidean distances of Nei's D and using (a.) single linkage, (b.) average linkage and (c.) Ward criterion clustering algorithm. The S (= path length) value stands for the sum of pairwise distances between row neighbors. Population numbers given on the right and top correspond to numbers in Table Appendix 1. The darker the color, the lower the genetic differentiation between two populations.

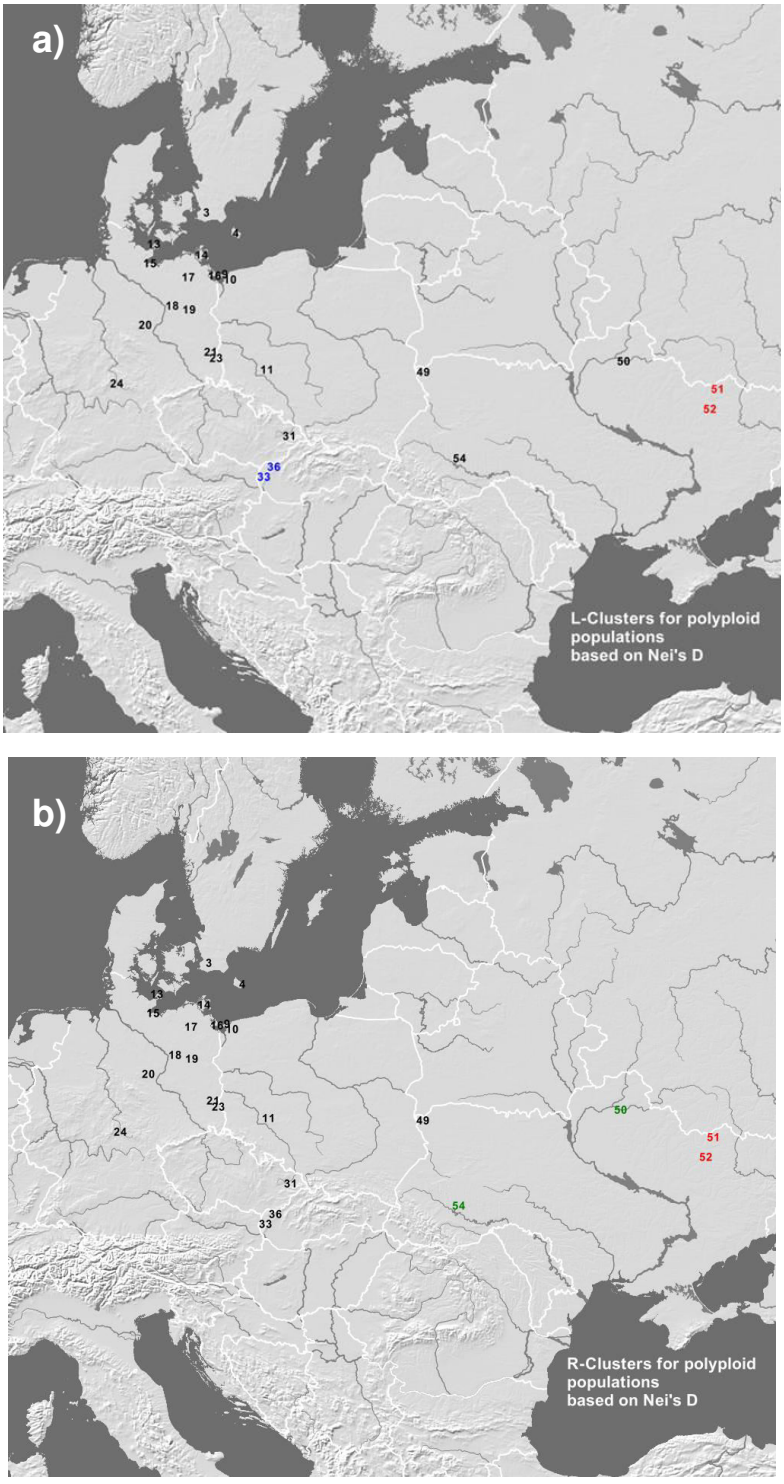

Figure 7: Genetic structuring of mixed-ploidy populations resulting from cluster analyses based on pairwise genetic distances (Nei's D) of microsatellite genotypes for a) the L genome and b) the R genome. Same colors indicate associations within a cluster. Black and red indicate the two main clusters found for both genomes; blue in a) and green in b) denote distinct subclusters within the black cluster for the L- and R-genome, respectively. For more details on population numbers see Appendix 1, for clustering details see Figures 5 and 6.

Genetic diversity and structure: mtDNA

We sequenced 1175 samples from 105 populations, which yielded a total of 75 haplotypes. The *lessonae*-specific ND2 + ND3 sequences exhibited 30 variable sites (25 in ND2 and 5 in ND3), which resulted in 40 haplotypes (Appendix 3). Nucleotide diversity (Pi) among *ridibundus* haplotypes was 0.0031 ± 0.0007. Overall, mean genetic distance was 0.31% among *lessonae* haplotypes (range: 0.82% to 0.07%). *Ridibundus*-specific ND2 + ND3 sequences exhibited 35 variable sites (29 in ND2 and 6 in ND3), which resulted in 32 haplotypes (Appendix 4). Among these, nucleotide diversity (Pi) was 0.0068 ± 0.0012, and overall mean genetic distance amounted to 0.67% (range: 1.57% to 0.07%). Most samples carried haplotypes that were *lessonae*- (n = 806, 68.6% of samples) or *ridibundus*-specific (n = 343, 30.0%), but we also found two haplotypes of mtDNA specific to *P. bergeri* (n = 15, 1.3%), and one haplotype that could be assigned to an Anatolian clade of *P.* cf. *bedriagae* (n = 1). Figure 8 gives an overview of the distribution of *lessonae*- and *ridibundus*-specific mtDNA found in the selected populations with respect to assigned population type.

The best fit-model of sequence evolution for the concatenated ND2 and ND3 sequences was the Hasegawa-Kishino-Yano-85 (HKY) model (Hasegawa et al. 1985) with a gamma-distribution (G) for site mutation rate (shape parameter = 0.529) and correction for invariant characters (I = 0.470). The maximum likelihood analysis yielded significant differentiation between *lessonae*-, *ridibundus*-, *bergeri*- and the Anatolian cf. *bedriagae*-type of mtDNA (Figure 9). Within the *lessonae*-group, differentiation was generally low, and only two clusters were supported by bootstrap values > 80. Polyploid individuals (LLR, LRR) were found in both clusters with a total of 16 haplotypes. Eight *lessonae*-haplotypes (from both clusters) were only found in *P. lessonae* individuals, and seven haplotypes from cluster les-1 were found also in *P. ridibundus* – apart from their occurrence in hybrids and *P. lessonae*.

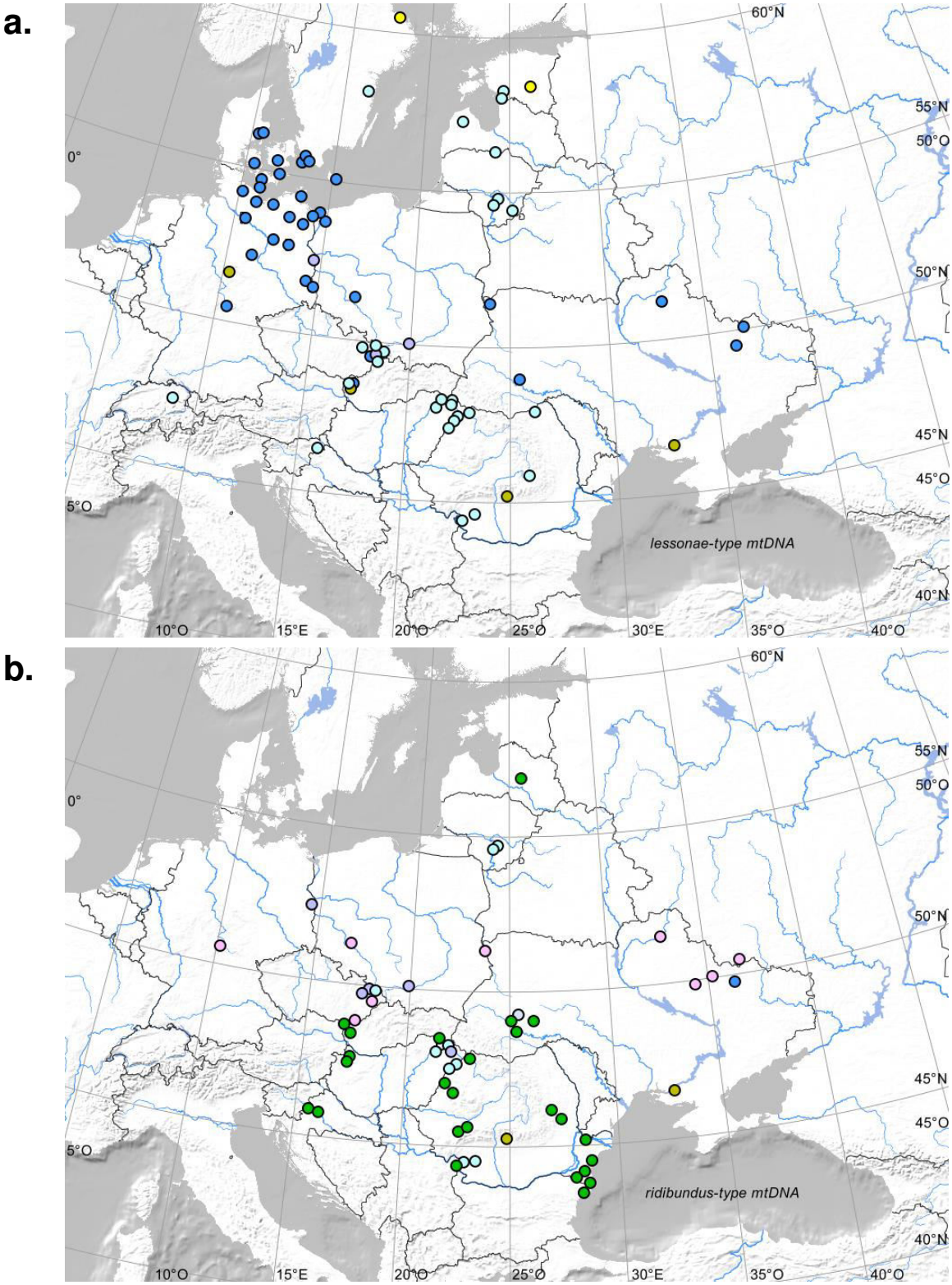

Figure 8: Sample origins of mtDNA types a.) *lessonae* and b) *ridibundus*. Symbol colors refer to population types: yellow = all LL; green = all RR; turquoise = diploid L-E; lilac = diploid R-E; mustard = diploid L-E-R; dark blue = mixed-ploidy with mtDNA type found in all genotypes including triploids; pink = mixed-ploidy with mtDNA type found only in LR and RR, but not in triploids.

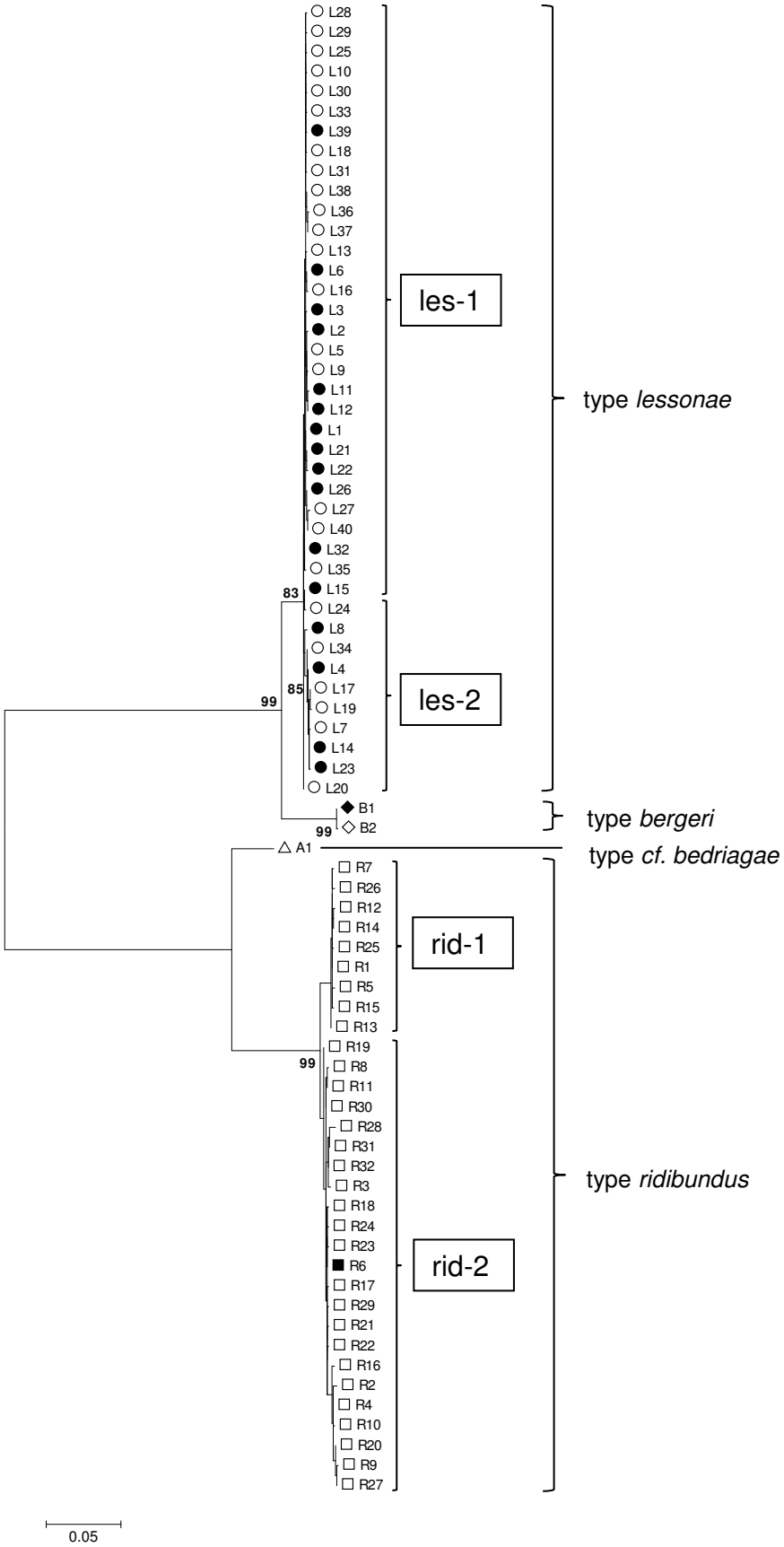

Figure 9: Phylogenetic relationships among 75 mtDNA haplotypes, as inferred from Maximum Likelihood Analysis. The units on the scale bar are expected mutations per site. Filled symbols indicate haplotypes found in polyploids. Values higher than 95 are considered statistically significant. Bootstrap values below 80 are not shown.

The *ridibundus*-group showed a significant differentiation into two clusters (Figure 9). Cluster rid-1 yielded one very common haplotype that occurs across several countries in both *P. esculentus* and *P. ridibundus*. The clusters contained eight other haplotypes that occurred in *P. ridibundus* specimen from Hungary, Slovakia, Croatia, and Bulgaria, as well as in two LR individuals from population [52]. In the second cluster, haplotypes found exclusively in *P. ridibundus* dominated, but, compared to the first cluster, more hybrid carriers of *ridibundus*-haplotypes were found. Within cluster two, also one very common *ridibundus*-haplotype (R6) was detected for the first time in polyploid individuals. Despite the wide distribution of R6 covering a large area and number of populations, this haplotype has not been found in polyploid individuals anywhere else than in five LRR individuals from population [52].

Discussion

Genetic diversity as a function of latitude, longitude and occurrence of parental species

Our study shows substantial geographical overlap among the different population types (Figure 1). Mixed-ploidy hybrid populations are rather common in north-central Europe up to southern Sweden but do not occur further south than to the rims of the Carpathian Mountains. Diploid hybrid populations were common in central Europe, pure *P. ridibundus* dominated south of the eastern Alps and of the Carpathians, and pure populations of *P. lessonae* were found only at two remote localities in Sweden and Latvia. This pattern widely confirms the distribution of the continental water frog species as reviewed and described by Plötner (2005). The impression that L-E populations often occur close to mountain ranges like the Alps, the Carpathians or the Harz (Central Germany), and pure *P. ridibundus* populations are more numerous close to large rivers and coastal areas of the Black Sea may thus be both a reflection of where we sampled and also of the actual habitat preferences (Günther 1990, Plötner 2005). A sampling bias may be responsible for the scarcity of mixed-ploidy populations in the Eastern Ukraine where we had samples from only a few localities, but polyploids are known to be very common (Borkin et al. 2004). Sampling sites from this highly interesting area are thus underrepresented in our study. On the other hand, the high density of mixed-ploidy populations across a defined area stretching from Southern Sweden to Germany and into Poland in our sample is surely representative for the real distribution of mixed-ploidy populations of the E-E system (Christiansen et al. 2005, Arioli et al. 2010, Christiansen and Reyer 2011). The low density of mixed-ploidy populations in the east-central part of Europe just slightly north of the gap between the Alps and the Carpathian range probably reflects a true scarcity of this population type in this area. The three localities in the Czech Republic [31] and Slovakia ([33],[36]), where we found triploid LLR individuals, basically cover the area where polyploids have been reported in these countries (Mikulicek and Kotlík 2001, Pruvost 2013). We failed

to find polyploid frogs in Hungary, although we sampled at localities ([46], [47]) close to the area where LLR individuals were documented two decades ago by (Tunner and Heppich-Tunner 1992). Instead, we found exclusively *P. ridibundus* in these localities. Possible explanations for this could be that we either did not sample in the right spot, or that environmental conditions in the area have changed in a way that *P. ridibundus* gained a survival advantage over *P. esculentus* hybrids, which can lead to the (local) decline of polyploid hybrids (Christiansen 2009).

Genetic diversity in our sample was generally higher in the R genome than in the L genome. This is probably attributable to the dominance of all-ridibundus populations (20 versus 2 all-lessonae) among the pure parental populations in our sample and, hence, a higher effective population size n_e. The population size effect is further supported by the fact, that the highest genetic diversity values for the R genome (HeR) were found in Southeastern Europe, where pure populations of *P. ridibundus* are very common. Pure *P. lessonae* populations, on the other hand, are rare (Plötner 2005) and seem to occur only in the marginal northernmost parts of the pool frog's distribution area, where they show low genetic variation (Sjögren 1991, Sjögren-Gulve and Berg 1999). In both the L- and R-genomes of *P. esculentus*, genetic diversity decreased with higher latitude, despite differences in distribution and frequency of sympatry between hybrids and parental species.

According to the postglacial refuge theory, rapid northwards expansion following interglacials often resulted in low genetic diversity caused by repeated founder effects that led to a loss of alleles and heterozygosity (Hewitt 1999). Rapid long-distance northward dispersal at low altitudes and along river valleys during warm climate periods appear likely for water frogs (Zeisset and Beebee 2001). In the case of such rapid expansions, few long-distance dispersers can lead to successful founding events that inhibit the establishment of later arriving genotypes. This phenomenon is also known as high-density blocking (Waters et al. 2013). In the case of the mixed-ploidy all-hybrid populations in Northern and North-Central Europe, however, we can assume that their success is also the

result of a continuous competitive advantage over their parental species, whether they had a historical head start or not. Christiansen (2009) demonstrated in a data-based equilibrium model that both parental genotypes would dominate (in the case of RR) or even drive all-hybrid populations to extinction (in the case of LL) after less than 40 generations if their survival rates were higher than those of the hybrids. Since we know that at least *P. lessonae* succeeded in expanding its postglacial range to high latitudes (Sjögren 1991, Sjögren-Gulve and Berg 1999, Zeisset and Beebee 2001), the dominance of *P. esculentus* in Northern and North-Central Europe thus suggests some continuous competitive advantages of the hybrid during the colonization of the areas formerly covered by glaciers. One competitive advantage in Northern regions with shorter and cooler summers could be that *P. esculentus* hybrids develop faster and perform better at colder temperatures during their larval stages than parental genotypes (Negovetics et al. 2001, Pruvost et al. 2013). Since hybrid water frog females produce large quantities of eggs (Berger and Uzzell 1980), hybrid offspring may quickly outnumber the less fecund *P. lessonae* and - when the conditions favor the hybrids' survival – event the more fecund *P. ridibundus*. Sometimes this is compensated by low reproductive success of hybrid males in mixed populations with *P. lessonae* (Abt and Reyer 1993), but matings between hybrids result in more viable offspring in mixed-ploidy populations of the E-E system, than in hybridogenetic diploid systems, because of the polyploids' ability to recombine homologous genomes (Günther et al. 1979, Christiansen and Reyer 2009). Further competitive advantages of hybrid over parental genotypes may have arisen from repeated primary hybridization in the suture zone of *P. lessonae* and *P. ridibundus*, which led to different hybrid lineages (Hotz et al. 2008) that provide the basis for local adaptation and selection among hybrid haplotypes (Pagano et al. 2008).

In sum, the success of polyploid all-hybrid populations across large, connected distribution areas - as the range of the E-E system of north-central Europe - is probably the result of a combination of a genetically based reproductive independence from the parental species and ecologically

determined competitive advantages. In contrast, in most regions that are further south, where diploid hybrid or pure parental (RR) systems prevail, genetic mechanisms leading to polyploidy may not have arisen and/or polyploids do not have gained a competitive advantage over the parental genotypes. Where polyploids do occur in these areas, they usually co-exist with *P. lessonae* or *P. ridibundus*.

Genetic distance between polyploid populations

We found that genetic differentiation among mixed-ploidy populations was generally high. On the one hand, this can be attributed to isolation by distance, which our data supported for all water frog populations, irrespective of their hybrid status or ploidy. On the other hand, polyploidy could theoretically have added to the degree of genetic differentiation. In plants, polyploidy can induce novel gene combinations through homologous recombination (Gaeta and Pires 2009) which can be advantageous, provided that stable chromosomal inheritance is established in the population through natural selection. In order to observe strong differentiation among populations caused by polyploidization, this would require at least several of such events and subsequent isolation between mixed-ploidy populations.

We performed hierarchical clustering analyses based on microsatellite data to identify a structure of genetic similarity among mixed-ploidy populations. Although microsatellites are not the best tool to infer phylogenetic relationships, they are useful for exploring genealogical relationships among populations. By comparing different clustering methods, we could gather a fairly congruent pattern for genetic distances in the R genome and, to a slightly lesser extent, in the L genome. In general, genetic distance values between populations containing polyploids were higher in the R genome than in the L genome. Cluster differentiation between mixed-ploidy populations was more pronounced in the R genome than in the L genome, but results were similar in the sense that for both genomes all three cluster algorithms we used identified two main clusters. These main clusters separated two populations from East Ukraine ([51],[52]) from the

majority of Central European populations. Hybrids in these two populations are thus genetically set off from other mixed-ploidy populations in both parental genome, while most of the other mixed-ploidy populations appear not as highly differentiated to support a robust genealogical pattern. However, within the main clusters, we found one consistent subcluster in each genome. For the R genome, this consisted of populations ([50] and [54]), which are situated in Western Ukraine, about half-way between the Central European populations and the populations [51] and [52]. For the L genome, populations [33] and [36] from Slovakia not only geographically fell right in between the most Western and most Eastern populations, but also genetically they formed a “bridging” cluster, since assortment to one or the other main cluster was not consistent among algorithms. Whatever the exact genetic association, we have other reasons to expect that these two nearby populations [33] and [36] should be genetically particular, since their gamete production pattern and breeding system differ substantially from those in Northern E-E population systems (Pruvost et al. 2013). In these two populations, polyploids occur only as male LLR. These produce diploid clonal LL gametes, rather than recombined haploid L gametes as their counterparts in northern E-E-systems do (Tunner 1994, Mikulicek and Kotlík 2001, Pruvost et al. 2013). We originally had expected a third population [31] to genetically associate with populations [33] and [36] , since triploid LLR males are also common in this population, show the same pattern of gamete production (Pruvost et al. 2013) and have been shown to be genetically similar to populations [33] and [36] in a different study (Pruvost 2013). The fact that we did not find the expected association could be explained by a slightly differing set of microsatellite markers that we used in the present study and by the generally weak power of clustering based on only four L-genome-specific markers. For both genomes, subclustering among the rest of populations was not congruent between the different methods and we thus did not attempt a more detailed interpretation of the clustering patterns. We assume that the smaller clusters we found in our study consist of populations that are closely connected by geographic proximity or shared common routes of colonization, or even

phylogenetic origin. The question, whether genetic differentiation between genetically different hybrid clades will eventually lead to speciation among polyploid hybrids, remains still open. Speciation would require some degree of reproductive and/or ecological isolation. According to recent studies, there is little reproductive isolation between water frogs from different population systems and regions in North and Central Europe (Pruvost et al. 2013).

Patterns of mtDNA haplotypes

Plötner et al. (2008) previously investigated patterns of mtDNA transfer in European water frogs based on a large data set covering an area comparable to this study. They found that, while introgression of *ridibundus*-type mtDNA was never found in *P. lessonae*, the reverse pattern (i.e. occurrence of *P. ridibundus* specimen carrying introgressed *lessonae*-specific mtDNA) was common in Central Europe and closely correlated with sympatry of *P. esculentus*. In Eastern and Southeastern Europe, *P. ridibundus* exclusively carries *ridibundus*-specific mtDNA, irrespective of the occurrence of *P. esculentus* or *P. lessonae*. Our results confirm most of these findings, like the absence of rid-type mtDNA in *P. lessonae* and the common presence of introgressed *les*-type mtDNA in Central European *P. ridibundus*. Interestingly, hybrids appear to mirror this pattern. While *P. esculentus* carries *lessonae-specific* mtDNA in areas where presently no primary hybridization can occur because of the absence of *P. ridibundus*, diploid hybrids can carry both types in areas where both parental species occur and primary hybridization is possible (Spolsky and Uzzell 1986). This was also true in our study.

According to Plötner et al. (2008), triploid hybrids from mixed-ploidy populations always carried les-type mtDNA without any exception, but in the study they did not differentiate between LLR and LRR. For most polyploid individuals investigated in this study, our data confirm this. However, we identified one *ridibundus*-haplotype in five polyploid LRR frogs from a population in Eastern Ukraine. Previous studies observed that polyploidy (LLR and LRR, very rarely tetraploidy) in *P. esculentus* occurs at high quantities and across

different population systems (including mixed systems with *P. lessonae* and *P. ridibundus*, or both) in Ukraine and Russia (Borkin et al. 2004, Borkin et al. 2006), especially along the Donets River, a large fluvial area in Eastern Ukraine. It appears that these eastern populations of polyploid hybrids are similarly widespread and successful as the hybrids of the north-central European E-E system, although populations [51] and [52] indicate that they are genetically different. Since *P. esculentus* is the result of multiple hybridization events and its hybridogenetic lineages yield a diversity of hemiclones (Hotz et al. 2008, Pagano et al. 2008), it is very probable that polyploid lineages evolved several times independently, which would mean that the main population clusters we identified in our study have different origins. For other amphibian taxa, multiple origins of polyploid lineages are well documented (e.g. Ptacek et al. 1994, Holloway et al. 2006), thus giving support to the hypothesis that several hybridization events and regular interaction of parental genotypes, rather than one unique “accident”, may be the normal route leading to the successful establishment of polyploid lineages (Dowling and Secor 1997).

Introgression beyond breeding systems

Apart from the existence of different genetic clusters among mixed-ploidy populations, we also found some interesting cases of mitochondrial introgression from water frog species other than *P. lessonae* and *P. ridibundus* into *P. esculentus* population systems. MtDNA typical for the Italian pool frog *P. bergeri* has been previously reported to occur in *P. lessonae* and diploid *P. esculentus* from Switzerland and Southern Germany (Hotz et al. 1992, Plötner et al. 2008). In our study, *bergeri*-type mtDNA was found in the three westernmost populations, two of them being diploid populations ([40], Switzerland and [22], Central Germany) and one a mixed-ploidy population containing few RR individuals ([24], Central Germany). Whereas in the two diploid populations *bergeri*-specific mtDNA was carried both by *P. lessonae* and LR hybrids, in [24] (where *P. lessonae* is probably absent) we found *bergeri*-specific mtDNA only in diploid hybrids and – for the first time - in one triploid LRR-female. Our

microsatellite data show that the [24] population is - although not more genetically diverse than other German populations - very diverse in terms of ploidy types and types of mtDNA. This population contains both types of triploids, some *ridibundus* genotypes and three types of mtDNA: *lessonae, ridibundus* and *bergeri*. While the *lessonae*-type is typical for north-central all-hybrid populations, *ridibundus*-haplotypes are rare in Central European hybrid populations and are much more common further East. Finally, the *bergeri*-type has been documented only in Swiss and German L-E systems. Since [24] lies right in the center of Germany, it is possible that frogs from different breeding systems (and possibly different colonization routes) have encountered there and mutually exchanged parts of their DNA, which passed through various types of hybrids. Whether these frogs got there by themselves (e.g. along major rivers) or were brought there recently by humans, e.g. as larvae with fish ([24] is a fish breeding pond), remains to be investigated.

At the easternmost edge of our sampling range we found another particularly interesting novel record of heterospecific introgression of mtDNA into *P. esculentus*. For the first time, a diploid *P. esculentus* hybrid from [52] (Southeastern Ukraine) was identified to carry an Anatolian-type cf. *bedriagae*-specific mtDNA. A combination like this could come about when a hybrid *P. esculentus* male mated with a female *P.* cf. *bedriagae,* or when a *P. esculentus* male mated with a *P. ridibundus* female carrying cf. *bedriagae*-specific mtDNA as a heritage from an earlier hybridization event between *P. ridibundus* and *P. cf. bedriagae*. The first scenario should be less likely, since the distribution area of *P.* cf*. bedriagae* ranges from western Anatolia to the Caspian Sea (Akin et al. 2010). Therefore, we favor the scenario of a previous hybridization between *P. ridibundus* and *P.* cf*. bedriagae,* which could have taken place before *P. ridibundus* extended its range from the Black Sea northwards, since there is evidence for migration of water frogs from Anatolia into Europe and hybridization between Anatolian and European individuals, for example in eastern Greece (Hotz et al. 2013). All in all, we found evidence that the allopolyploid hybrid *P. esculentus* not only incorporated genetic information from its two original parental

species, but apparently succeeded to extend its genetic heritage to other water frog species living close to the respective distribution borders of *P. lessonae* (proximity to contact zone with *P. bergeri*, in our study: Switzerland and Germany) and *P. ridibundus* (the transition zones to Anatolian water frogs in eastern Greece and west of the Caspian Sea).

Conclusions

We suggest a scenario according to which hybridogenetic *P. esculentus* repeatedly resulted from primary hybridization between *P. lessonae* and hybridogenesis-inducing *P. ridibundus* genotypes in a postglacial suture zone southeast of the Alps and Carpathians. From there they probably expanded to Central and Northern Europe, following the northwestwards branch of a bifurcating colonization route that has been suggested for *P. lessonae* (Zeisset and Beebee 2001) and other European amphibians (Hewitt 1999, Stöck et al. 2012). Along the migration route, the first polyploid hybrids could have emerged through pairings between diploid parental or hybrid males and diploid hybrid females which, as a novelty, produced diploid eggs as it is characteristic of LR females from E-E- and many LE-system populations (Berger and Uzzell 1980, Graf and Polls Pelaz 1989, Arioli 2007, Czarniewska et al. 2011). Colder climates during the reproductive season could have played a role in inducing polyploidy during gametogenesis (Kawamura 1984, Kondo and Kashiwagi 2004). Other groups of hybrids probably extended their range from the Black Sea area towards the area of today's Ukraine, where strong genetic differentiation and novel introgression patterns of mtDNA suggest at least one more separate system of polyploid *P. esculentus* that might not share the same phylogenetic origin than the other polyploid populations in our study.

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Appendix 1: Geographic coordinates, sample sizes and genotypic composition of waterfrog populations sampled for microsatellite analysis. Classification was based on microsatellite results and was complemented by additional information on the population through personal observation (e.g. on presence of parental genotypes even though none were captured for sampling).

Nr.	Country	Population	Latitude	Longitude	n L	n R	N	% LR	% RR	% LL	% LLR	% LLRR	% LLRR	HeL	HeR	classification
1	Sweden	Uppsala	60°33'24.52"N	17°51'46.04"E	20		20	0	0	100	0	0	0	0.19		all LL
2	Sweden	Östergötland	58°5'38.59"N	16°22'28.68"E	23	12	23	52.17	0	47.83	0	0	0	0.12	0	diploid L-E
3	Sweden	Genarp	55°36'34.00"N	13°23'19.00"E	63	80	80	70	0	0	11.25	18.75	0	0.15	0.09	mixed-ploidy
4	Denmark	Bornholm	55°7'28.72"N	15°8'58.57"E	32	38	38	47.37	13.16	0	7.89	26.32	5.26	0.13	0.41	mixed-ploidy
5	Estonia	Laeva	58°25'41.99"N	26°19'7.99"E	20		20	0	0	100	0	0	0	0.29		all LL
6	Latvia	Stickli	57°19'41.00"N	22°15'21.99"E	20		20	30	0	70	0	0	0	0.31		diploid L-E
7	Latvia	Jurmala	56°59'36.99"N	25°55'22.00"E		14	14	0	100	0	0	0	0		0.48	all RR
8	Lithuania	Baltoji Voke	54°28'44.00"N	25°7'59.00"E	24	12	24	50	0	50	0	0	0	0.32	0.31	diploid L-E
9	Poland	Kolczewo	53°57' 54.0"N	14°36'34.10"E	7	7	7	71.43	0	0	14.29	14.29	0	0.05	0.32	mixed-ploidy
10	Poland	Wysoka	53°49'53.47"N	14°51'51.53"E	37	37	37	54.05	0	0	35.14	10.81	0	0.21	0.44	mixed-ploidy
11	Poland	Sanie	51°25'38.10"N	16°56'57.84"E	54	48	61	62.3	8.2	21.31	1.64	3.28	3.28	0.39	0.29	mixed-ploidy
12	Poland	Krakow	50°5'3.60"N	19°50'26.46"E		12	12	0	100	0	0	0	0		0.51	all RR
13	Germany	Fehmarn	54°31'9.984"N	11°3'13.96"E	26	26	26	65.38	0	0	34.62	0	0	0.17	0.24	mixed-ploidy
14	Germany	Rügen	54°25'1.44"N	13°23'45.39"E	16	16	16	56.25	0	0	43.75	0	0	0.26	0.25	mixed-ploidy
15	Germany	Klützer Winkel	53°59'23.34"N	11°0'37.02"E	26	26	26	30.77	0	0	38.46	30.77	0	0.25	0.33	mixed-ploidy
16	Germany	Usedom	53°52'44.82"N	14°8'20.58"E	21	21	21	33.33	0	0	57.14	9.52	0	0.27	0.49	mixed-ploidy
17	Germany	Grammentin	53°45'26.72"N	12°53'41.04"E	19	19	19	21.05	0	0	36.84	42.11	0	0.22	0.29	mixed-ploidy
18	Germany	Schönermark	52°54'7.08"N	12°19'15.50"E	79	78	79	46.84	0	1.27	15.19	36.71	0	0.32	0.39	mixed-ploidy
19	Germany	Teschendorf	52°51'53.03"N	13°8'40.38"E	34	33	34	17.65	0	2.94	76.47	2.94	0	0.29	0.32	mixed-ploidy
20	Germany	Altenhausen	52°16'40.0"N	11°15'15.01"E	29	26	29	27.59	0	10.34	58.62	3.45	0	0.29	0.49	mixed-ploidy
21	Germany	Cottbus	51°46'24.30"N	14°21'19.14"E	42	49	49	40.82	14.29	0	16.33	28.57	0	0.11	0.45	mixed-ploidy
22	Germany	Herzberg	51°37'36.66"N	10°21'15.06"E	35	50	60	41.67	41.67	16.67	0	0	0	0.44	0.4	diploid L-E-R
23	Germany	Döbern	51°36'38.22"N	14°36'15.60"E	63	63	63	50.79	0	0	15.87	33.33	0	0.2	0.28	mixed-ploidy
24	Germany	Untermassfeld	50°32'21.06"N	10°24'28.44"E	39	44	44	61.36	11.36	0	0	25	2.27	0.38	0.43	mixed-ploidy
25	Czech Rep.	Břidličná	49°55'00.40"N	17°21'39.80"E	35	31	35	88.57	0	11.43	0	0	0	0.43	0.29	diploid L-E
26	Czech Rep.	Nový Stav	49°52'38.52"N	18°21'23.04"E	33	44	44	75	25	0	0	0	0	0	0.57	diploid R-E
27	Czech Rep.	Zpupna Lhota	49°45'42.06"N	18°35'54.42"E	43	43	43	100	0	0	0	0	0.45	0.27		diploid L-E
28	Czech Rep.	Albrechtičky	49°42'18.36"N	18°04'51.30"E	3	16	16	18.75	81.25	0	0	0	0		0.59	diploid R-E
29	Czech Rep.	Trnávka	49°40'54.90"N	18°11'5.22"E	54	44	58	68.97	6.9	24.14	0	0	0	0.41	0.17	diploid L-E
30	Czech Rep.	Dobrá	49°40'36.96"N	18°23'30.18"E	55	24	55	43.64	0	56.36	0	0	0	0.47	0.18	diploid L-E
31	Czech Rep.	Borovec	49°38'8.50"N	18°6'1.90"E	46	65	65	64.62	29.23	0	6	0	0	0.18	0.19	mixed-ploidy
32	Slovakia	Brodské	48°41'37.11"N	17°0'29.93"E		15	15	0	100	0	0	0	0		0.59	all RR
33	Slovakia	Šajdíkove Humen	48°39'14.30"N	17°17'1.19"E	29	32	32	37.5	9.38	0	53.13	0	0	0.34	0.35	mixed-ploidy
34	Slovakia	Kalaštov	48°37'55.26"N	17°15'12.30"E	35	25	35	91.43	0	8.57	0	0	0	0.42	0.34	diploid L-E
35	Slovakia	Šaštín-Stráže	48°37'54.61"N	17°8'40.38"E	62	70	86	53.49	27.91	18.6	0	0	0	0.47	0.46	diploid L-E-R
36	Slovakia	Kozi chrbát	48°37'53.58"N	17°17'41.28"E	67	67	67	43.28	0	0	56.72	0	0	0.39	0.36	mixed-ploidy
37	Slovakia	Borský Mikuláš	48°37'45.60"N	17°11'17.34"E	39	24	39	61.54	0	38.46	0	0	0	0.46	0.34	diploid L-E
38	Slovakia	Lakšárska	48°33'39.40"N	17°10'1.7"E	26	25	26	96.15	0	3.85	0	0	0	0.47	0.4	diploid L-E
39	Slovakia	Šprinclov Majer	48°12'59.85"N	17°11'15.51"E		10	10	0	100	0	0	0	0		0.47	all RR
40	Switzerland	Hellberg	47°17'45.72"N	8°48'48.38"E	14		14	21.43	0	78.57	0	0	0	0.69		diploid L-E
41	Hungary	Zempleńi-hegpég	48°20'13.08"N	2139'04.80"E	14	15	15	93.33	6.67	0	0	0	0	0.3	0.12	diploid R-E
42	Hungary	Sátoraljaújhely	48°20'10.40"N	21°39'11.90"E	7	6	7	85.71	0	14.29	0	0	0	0.38	0.2	diploid L-E
43	Hungary	Szabolsveresmat	48°17'32.04"N	22°1'57.65"E	7	7	7	100	0	0	0	0	0	0.39	0.16	diploid L-E
44	Hungary	Kisvárda	48°13'50.37"N	22°3'39.53"E	9	9	9	100	0	0	0	0	0	0.43	0.19	diploid L-E
45	Hungary	Várkötzi-morotva	48°7'0.00"N	21°26'60.00"E	7	6	7	85.71	0	14.29	0	0	0	0.43	0.23	diploid L-E
46	Hungary	Kapuvar	47°40'3.60"N	17°8'2.70"E		61	61	0	100	0	0	0	0		0.57	all RR
47	Hungary	Osli	47°37'52.90"N	17°4'48.20"E		21	21	0	100	0	0	0	0		0.58	all RR
48	Hungary	Lakitelek	48°25'13.10"N	21°36'19.00"E		13	13	0	100	0	0	0	0		0.57	all RR
49	Ukraine	Shatsk	51°29'17.20"N	23°55'53.40"E	15	19	19	73.68	21.05	0	0	5.26	0	0.43	0.27	mixed-ploidy
50	Ukraine	Baturin	51°20'19.39"N	32°52'43.54"E	13	25	25	40	48	0	4	8	0	0.37	0.54	mixed-ploidy
51	Ukraine	Zhovtneve	50°8'3.25"N	36°45'58.65"E	19	22	22	77.27	13.64	0	9.09	0	0	0.16	0.52	mixed-ploidy
52	Ukraine	Gaidary Iskov	49°37'23.70"N	36°17'14.89"E	71	81	81	69.14	12.35	0	1.23	17.28	0	0.28	0.52	mixed-ploidy
53	Ukraine	Poltava	49°36'2.52"N	34°32'29.69"E		6	6	0	100	0	0	0	0		0.59	all RR
54	Ukraine	Buchach	49°3'52.70"N	25°22'59.16"E	17	20	26	38.46	34.62	23.08	3.85	0	0	0.4	0.68	mixed-ploidy
55	Ukraine	Zyurupinsk	46°36'50.80"N	32°43'10.13"E	5	21	23	13.04	78.26	8.7	0	0	0	0.25	0.65	diploid L-E-R
56	Ukraine	Vilkovo	45°23'58.16"N	29°35'42.18"E		6	6	0	100	0	0	0	0		0.77	all RR
57	Slowenia	Kicar	46°26'36.50"N	15°55'45.70"E	33	19	33	57.58	0	42.42	0	0	0	0.48	0.41	diploid L-E
58	Slowenia	Prilipe	45°52'42.80"N	15°37'31.30"E		43	43	0	100	0	0	0	0		0.73	all RR
59	Croatia	Zagreb	45°50'9.30"N	16°4'9.30"E		39	39	0	100	0	0	0	0		0.72	all RR
60	Croatia	Kutina	45°31'2.30"N	16°56'43.30"E		9	9	0	100	0	0	0	0		0.62	all RR
61	Romania	Oradea	47°3'46.35"N	21°56'12.74"E		5	5	0	100	0	0	0	0		0.64	all RR
62	Romania	Reci	45°50'28.68"N	25°55'48.39"E	8		8	37.5	0	62.5	0	0	0	0.45		diploid L-E
63	Romania	Ciopeia	45°33'11.16"N	22°58'17.04"E		24	24	0	100	0	0	0	0		0.64	all RR
64	Romania	Sarmizegetuza	45°30'4.68"N	22°46'6.60"E		23	23	0	100	0	0	0	0		0.64	all RR
65	Romania	Nucşoara	45°20'15.54"N	24°46'55.64"E	7	5	7	14.29	57.14	85.71	0	0	0	0.4	0.65	diploid L-E-R
66	Romania	Argineşti	44°34'32.38"N	23°24'47.96"E	7		7	28.57	0	71.43	0	0	0	0.42		diploid
67	Romania	Sinoe	44°33'40"N	28°45'48"E		10	10	0	100	0	0	0	0		0.79	all RR
68	Romania	Hinova	44°32'23.00"N	22°46'38.00"E	5	5	5	100	0	0	0	0	0		0.29	diploid L-E
69	Romania	Scăpău	44°27'39.24"N	22°43'29.39"E		5	5	0	100	0	0	0	0		0.78	all RR
70	Romania	Basarabi	44°10'48"N	28°24'36.3"E		9	9	0	100	0	0	0	0		0.8	all RR
71	Bulgaria	Durankulak	43°42'2.88"N	28°34'31.80"E		14	14	0	100	0	0	0	0		0.67	all RR
72	Bulgaria	Bolata Dere	43°23'37.68"N	28°27'58.68"E		7	7	0	100	0	0	0	0		0.69	all RR

Appendix 2: Populations, geographic coordinates and sample sizes of samples used for mtDNA analysis. The column "Microsat.Nr" refers to the numbering of populations in Table 2, where only populations used in microsatellite analyses are listed. Types of mtDNA are abbreviated as follows: les = *lessonae* -type, rid = *ridibundus* -type, ber = *bergeri* -type, cf. bed = cf. *bedriagae* -type.

Nr.	Microsat Nr.*	Country	Population	Latitude	Longitude	N	n type mitochondrial DNA			
							les	rid	berg	cf. bed
SE-1	1	Sweden	Uppsala	60°33'24.52"N	17°51'46.04"E	13		13		
SE-2	2	Sweden	Östergötland	58°5'38.59"N	16°22'28.68"E	11		11		
SE-3	3	Sweden	Genarp	55°36'34.00"N	13°23'19.00"E	16		16		
SE-4		Sweden	Skane 001	55°35'17.80"N	13°21'07.67"E	3		3		
SE-5		Sweden	Skane 032	55°34'03.00"N	13°12'53.00"E	4		4		
SE-6		Sweden	Skane 050	55°29'33.00"N	13°08'02.00"E	5		5		
SE-7		Sweden	Skane 159	55°23'17.04"N	13°26'50.99"E	5		5		
DK-1		Denmark	Jutland	56°09'02.99"N	10°31'57.00"E	33		33		
DK-2	4	Denmark	Bornholm	55°07'28.72"N	15°08'58.57"E	31		31		
DK-3		Denmark	Sjelland	55°27'47.71"N	11°43'17.39"E	4		4		
DK-4		Denmark	Funen	55°07'09.00"N	10°30'30.00"E	15		15		
DK-5		Denmark	Saeland	55°00'30.99"N	12°00'16.99"E	28		28		
DK-6		Denmark	Lolland	54°47'30.00"N	11°00'11.00"E	16		16		
EE-1	5	Estonia	Laeva	58°25'41.99"N	26°19'7.99"E	12		12		
EE-2		Estonia	Pärnu	58°23'05.00"N	24°31'07.00"E	1		1		
EE-3		Estonia	Hara	58°05'16.01"N	24°29'39.00"E	11		11		
LV-1	6	Latvia	Stickli	57°19'41.00"N	22°15'21.99"E	8		8		
LV-2	7	Latvia	Jurmala	56°59'36.99"N	25°55'22.00"E	6	6			
LT-1		Lithuania	LT-B	56°22'52.00"N	24°10'06.00"E	4		4		
LT-2		Lithuania	LT-K	54°47'49.00"N	24°15'05.00"E	13	7	6		
LT-3		Lithuania	Dasunikeskes	54°42'49.00"N	24°05'54.00"E	2	1	1		
LT-4	8	Lithuania	Baltoji Voke	54°28'44.00"N	25°7'59.00"E	23		23		
PL-1	9	Poland	Kolczewo	53°57'54.0"N	14°36'34.10"E	8		8		
PL-2	10	Poland	Wysoka	53°49'53.47"N	14°51'51.53"E	25		25		
PL-3	11	Poland	Sanie	51°25'38.10"N	16°56'57.84"E	49	4	45		
PL-4	12	Poland	Krakow	50°5'3.60"N	19°50'26.46"E	11	1	10		
D-1	13	Germany	Fehmarn	54°31'9.984"N	11°3'13.96"E	13		13		
D-2	14	Germany	Rügen	54°25'1.44"N	13°23'45.39"E	16		16		
D-3		Germany	Preetz	54°14'50.30"N	10°11'26.63"E	2		2		
D-4		Germany	Hanstorf	54°02'26.96"N	11°54'40.20"E	2		2		
D-5	15	Germany	Klützer Winkel	53°59'23.34"N	11°0'37.02"E	14		14		
D-6	16	Germany	Usedom	53°52'44.82"N	14°8'20.58"E	8		8		
D-7	17	Germany	Grammentin	53°45'26.72"N	12°53'41.04"E	5		5		
D-8		Germany	Rothemühl	53°34'26.00"N	13°46'04.00"E	2		2		
D-9		Germany	Gülzow	53°26'41.14"N	10°30'04.72"E	6		6		
D-10	18	Germany	Schönermark	52°54'7.08"N	12°19'15.50"E	2		2		
D-11		Germany	Dörverden	52°53'47.00"N	9°16'26.00"E	15		15		
D-12	19	Germany	Teschendorf	52°51'53.03"N	13°8'40.38"E	10		10		
D-13		Germany	Lebus	52°24'50.76"N	14°32'31.92"E	7	2	5		
D-14	20	Germany	Altenhausen	52°16'40.0"N	11°15'15.01"E	9		9		
D-15	21	Germany	Cottbus	51°46'24.30"N	14°21'19.14"E	43		43		
D-16	22	Germany	Herzberg	51°37'36.66"N	10°21'15.06"E	7		2	5	
D-17	23	Germany	Döbern	51°36'38.22"N	14°36'15.60"E	46		46		
D-18	24	Germany	Untermassfeld	50°32'21.06"N	10°24'28.44"E	39	2	32	5	
CZ-1	25	Czech Rep.	Břidličná	49°55'00.40"N	17°21'39.80"E	5		5		
CZ-2	26	Czech Rep.	Nový Stav	49°52'38.52"N	18°21'23.04"E	24	19	5		
CZ-3	27	Czech Rep.	Zpupna Lhota	49°45'42.06"N	18°35'54.42"E	22		22		
CZ-4	28	Czech Rep.	Albrechtičky	49°42'18.36"N	18°04'51.30"E	5	5			
CZ-5	29	Czech Rep.	Trnávka	49°40'54.90"N	18°11'5.22"E	19	2	17		
CZ-6	30	Czech Rep.	Dobrá	49°40'36.96"N	18°23'30.18"E	39		39		
CZ-7	31	Czech Rep.	Borovec	49°38'8.50"N	18°6'1.90"E	30	4	26		
SK-1	32	Slovakia	Brodské	48°41'37.11"N	17°0'29.93"E	5	5			
SK-2	33	Slovakia	Šajd. Humence	48°39'14.30"N	17°17'1.19"E	12	1	11		
SK-3	34	Slovakia	Kalaštov	48°37'55.26"N	17°15'12.30"E	10		10		
SK-4	35	Slovakia	Šaštin-Stráže	48°37'54.61"N	17°8'40.38"E	3		3		
SK-5	36	Slovakia	Kozi chrbát	48°37'53.58"N	17°17'41.28"E	10		10		
SK-6	38	Slovakia	Lakšárska	48°33'39.40"N	17°10'1.7"E	4		4		
SK-7	39	Slovakia	Šprinclov Majer	48°12'59.85"N	17°11'15.51"E	9	9			
CH-1	40	Switzerland	Hellberg	47°17'45.72"N	8°48'48.38"E	5		2	3	
HUN-1	41	Hungary	Zempleńi-hegpég	48°20'13.08"N	2139'04.80"E	15	2	13		
HUN-2	42	Hungary	Sátoraljaújhely	48°20'10.40"N	21°39'11.90"E	7	2	5		
HUN-3	43	Hungary	Szabolsveresmat	48°17'32.04"N	22°1'57.65"E	6	1	5		
HUN-4	44	Hungary	Kisvárda	48°13'50.37"N	22°3'39.53"E	9	5	4		
HUN-5	45	Hungary	Várkötzi-morotva	48°7'0.00"N	21°26'60.00"E	7	2	5		
HUN-6	46	Hungary	Kapuvar	47°40'3.60"N	17°8'2.70"E	27	27			
HUN-7	47	Hungary	Osli	47°37'52.90"N	17°4'48.20"E	12	12			
HUN-8	48	Hungary	Lakitelek	48°25'13.10"N	21°36'19.00"E	12	12			
UA-1	49	Ukraine	Shatsk	51°29'17.20"N	23°55'53.40"E	16	9	7		
UA-2	50	Ukraine	Baturin	51°20'19.39"N	32°52'43.54"E	8	6	2		
UA-3	51	Ukraine	Zhovtneve	50°8'3.25"N	36°45'58.65"E	9	9			
UA-4	52	Ukraine	Gaidary Iskov Yar	49°37'23.70"N	36°17'14.89"E	46	45			1
UA-5	53	Ukraine	Poltava	49°36'2.52"N	34°32'29.69"E	4	4			
UA-6	54	Ukraine	Buchach	49°3'52.70"N	25°22'59.16"E	4	4			
UA-7	55	Ukraine	Zyurupinsk	46°36'50.80"N	32°43'10.13"E	2	2			
UA-8	56	Ukraine	Vilkovo	45°23'58.16"N	29°35'42.18"E	6	6			
SL-1	57	Slowenia	Kicar	46°26'36.50"N	15°55'45.70"E	12		12		
SL-2	58	Slowenia	Prilipe	45°52'42.80"N	15°37'31.30"E	9	9			
HR-1	59	Croatia	Zagreb	45°50'9.30"N	16°4'9.30"E	32	32			
ROM-1		Romania	Dersca	47°59'21.71"N	26°12'48.10"E	2		2		
ROM-2		Romania	Livada	47°51'54.63"N	23°07'10.47"E	3	1	2		
ROM-3		Romania	Foieni	47°41'53.70"N	22°23'14.07"E	4	3	1		
ROM-4		Romania	Resighea	47°35'49.66"N	22°17'47.35"E	4		4		
ROM-5		Romania	Şimian	47°29'21.00"N	22°05'17.20"E	5	3	2		
ROM-6	61	Romania	Oradea	47°03'46.35"N	21°56'12.74"E	4	4			
ROM-7		Romania	C. de Pomezeu	46°48'02.08"N	22°19'00.22"E	4	4			
ROM-8		Romania	Căiuţi	46°11'00.10"N	26°55'42.95"E	4	4			
ROM-9		Romania	Tecuci	45°50'48.03"N	27°26'05.82"E	3	3			
ROM-10	62	Romania	Reci	45°50'28.68"N	25°55'48.39"E	7		7		
ROM-11	63	Romania	Ciopeia	45°33'11.16"N	22°58'17.04"E	9	9			
ROM-12	64	Romania	Sarmizegetuza	45°30'04.68"N	22°46'06.60"E	10	10			
ROM-13	65	Romania	Nucşoara	45°20'15.54"N	24°46'55.64"E	8	4	4		
ROM-14		Romania	Saon	45°13'04.79"N	28°32'33.85"E	3	3			
ROM-15	66	Romania	Argineşti	44°34'32.38"N	23°24'47.96"E	7		7		
ROM-16		Romania	Histria	44°34'21.17"N	28°42'47.34"E	3	3			
ROM-17	67	Romania	Sinoe	44°33'40.00"N	28°45'48.00"E	7	7			
ROM-18	68	Romania	Hinova	44°32'23.00"N	22°46'38.00"E	5	3	2		
ROM-19	69	Romania	Scăpău	44°27'39.24"N	22°43'29.39"E	5	5			
ROM-20	70	Romania	Basarabi	44°10'48.00"N	28°24'36.3"E	8	8			
ROM-21		Romania	Furnica	43°57'36.67"N	28°00'07.46"E	3	3			
ROM-22		Romania	Mangalia	43°48'59.16"N	28°34'47.11"E	2	2			
BG-1	71	Bulgaria	Durankulak	43°42'2.88"N	28°34'31.80"E	9		9		
BG-2	72	Bulgaria	Bolata Dere	43°23'37.68"N	28°27'58.68"E	8	8			
					Total	**1175**	**344**	**817**	**13**	**1**

Appendix 3: Variable sites in the ND2 and ND3 gene and nucleotide composition among 40 haplotypes of *lessonae*-type mtDNA.

	Site (position) ND2																								ND3					
																						1	1	1	1	1	1	1	1
	1	1	2	3	3	4	4	4	4	5	6	6	6	7	7	8	8	8	8	8	9	9	0	0	1	1	1	1	2	2
	1	7	2	6	6	1	4	6	8	2	6	9	9	2	3	0	3	3	4	9	7	9	1	3	2	5	6	9	4	9
	5	7	1	6	9	5	5	3	9	0	1	1	2	1	8	7	2	5	0	2	5	6	1	6	3	8	7	7	2	3
L1	G	T	G	C	G	A	A	A	G	C	A	G	C	A	T	A	G	G	G	G	G	A	T	T	A	T	A	T	T	C
L2	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
L3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
L4	.	.	.	T	.	G	.	.	.	.	.	A	.	.	C	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.
L5	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
L7	.	.	.	T	.	G	.	.	.	.	.	A	.	.	C	G	A	.	A	.	.	.	.	.	.	.	G	.	.	.
L8	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	T
L9	.	.	.	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L10	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L11	.	.	.	.	A	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
L12	.	.	.	.	A	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L14	.	.	.	T	.	G	.	.	.	.	.	A	.	.	C	G	A	.	.	.	.	.	.	.	.	.	G	.	.	.
L15	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
L17	.	C	.	T	.	G	.	.	.	.	.	A	.	.	C	G	A	.	.	.	.	.	.	.	.	.	G	.	.	.
L18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
L19	.	C	.	T	.	G	.	.	.	.	.	A	.	.	C	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.
L20	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
L22	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
L23	.	.	.	T	.	G	.	.	.	.	.	A	.	.	C	G	A	.	.	.	.	.	.	G	.	.	G	.	.	.
L24	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
L25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
L26	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L27	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	G	.	.	.	.	.
L28	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
L29	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L30	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L31	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
L32	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L33	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L34	.	.	.	T	.	.	.	.	.	.	.	A	.	.	C	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.
L35	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L36	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
L37	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.
L38	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.
L39	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L40	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.

Appendix 4: Variable sites in the ND2 and ND3 gene and nucleotide composition among 32 haplotypes of *ridibundus*-type mtDNA.

	Site (position) ND2																												ND3					
																										1	1	1	1	1	1	1	1	1	1
		2	2	2	3	3	3	4	5	5	5	6	6	7	7	7	7	7	7	7	8	8	9	9	9	0	0	0	0	1	2	2	2	3	3
	3	2	3	7	2	4	4	7	0	7	9	2	5	1	2	2	3	4	4	6	6	9	6	6	7	0	0	1	2	1	0	8	9	1	1
	4	1	4	3	1	2	5	4	4	9	1	4	2	1	6	8	5	4	7	2	7	4	7	9	8	8	9	5	4	3	6	4	5	4	5
R1	A	G	T	T	T	A	C	C	G	G	G	C	T	C	T	C	C	G	G	G	A	T	G	C	T	A	C	A	C	C	A	C	T	T	G
R2	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	A	.	C	.	.	.	.	.	.	.	.	G	T	.	C	.
R3	.	.	.	C	.	G	.	T	.	A	A	T	.	T	.	.	.	.	A	.	.	C	.	.	.	.	.	.	.	T	.	T	C	C	.
R4	.	.	.	C	.	G	.	T	.	A	A	T	C	.	.	.	.	.	A	A	.	C	A	T	.	.	.	.	.	.	G	T	.	C	.
R5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R6	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	C	.	.	.	.	T	.	T	.	C	.
R7	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R8	C	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	C	.	.	.	.	T	.	T	.	C	.
R9	C	.	.	C	C	G	T	T	.	A	A	T	C	.	.	.	.	.	A	A	G	C	A	T	.	.	.	.	.	.	G	T	.	C	.
R10	C	.	.	C	.	G	.	T	.	A	A	T	C	.	.	.	.	.	A	A	.	C	A	T	.	.	.	.	.	.	G	T	.	C	.
R11	C	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	.	.	.	.	.	T	.	T	.	C	.
R12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.
R13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
R15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.
R16	.	.	.	C	.	G	.	T	.	A	A	T	C	.	.	.	.	.	A	A	.	C	A	T	.	.	.	.	.	.	.	.	.	.	.
R17	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	.	A	T	C	.	.	.	.	T	.	T	.	C	.
R18	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	C	.	.	.	.	T	.	T	.	C	A
R19	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	T	.	A	.	.	.	A	T	.	.	.	.	.	T	.	T	.	C	.
R20	.	.	.	C	C	G	T	T	.	A	A	T	C	.	.	.	.	.	A	A	.	C	A	T	.	.	.	.	.	.	G	T	.	C	.
R21	.	.	.	C	.	G	.	T	A	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	C	.	.	.	.	T	.	T	.	C	.
R22	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	C	.	T	.	.	T	.	T	.	C	.
R23	.	.	.	C	.	G	.	T	.	A	A	T	.	.	C	.	.	.	A	.	.	C	A	T	C	.	.	.	.	T	.	T	.	C	.
R24	.	A	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	C	.	.	.	.	T	.	T	.	C	.
R25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
R26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
R27	.	.	.	C	C	G	T	T	.	A	A	T	C	.	.	.	.	.	A	A	G	C	A	T	.	.	.	.	.	.	G	T	.	C	.
R28	.	.	.	C	.	G	.	.	.	A	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R29	.	.	C	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	C	.	.	.	.	T	.	T	.	C	.
R30	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	.	.	.	.	.	T	.	T	.	C	.
R31	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	T	.	C	.
R32	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	.	.	.	.	.	.	.	T	.	T	.	C	.

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Gamete production patterns, ploidy and population genetics reveal evolutionary significant units in hybrid water frogs (*Pelophylax esculentus*)

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Abstract

The European water frog *Pelophylax esculentus* is a natural hybrid between *P. lessonae* (genotype LL) and *P. ridibundus* (RR). It reproduces through hybridogenesis, eliminating one parental genome from its germline and producing gametes containing the genome of the other parental species. According to previous studies, this elimination and transmission pattern is very diverse. In mixed populations, where only diploid hybrids (LR) live in sympatry and mate with one or both parental species, the excluded genome varies among regions, and the remaining genome is transmitted clonally to haploid gametes. In all-hybrid populations consisting of diploid (LR) and triploid (LLR and/or LRR) frogs, diploid individuals also produce gametes clonally (1n in males, 2n in females), whereas triploids eliminate the genome they have in single copy and produce haploid gametes containing the recombined other genome. However, here, too, regional differences seem to exist, and some triploids have been reported to produce diploid gametes.

In order to systematically study such regional and genotype differences in gamete production, their potential origin, and their consequences for the breeding system, we sampled frogs from five populations in three European countries, performed crossing experiments and investigated the genetic variation through microsatellite analysis. For four populations, one in Poland, two in Germany and one in Slovakia, our results confirmed the elimination and transmission pattern described above. In one Slovakian population, however, we found a totally different pattern. Here, triploid males (LLR) produce sperm with a clonally transmitted diploid LL

genome, rather than a haploid recombined L genome, and LR females clonally produce haploid R eggs, rather than diploid LR eggs. These differences among the populations in gamete production go along with differences in genomotype composition, breeding system (i.e. the way triploids are produced) and genetic variation

These differences are strong evidence for a polyphyletic origin of triploids. Moreover, our findings shed light on the evolutionary potential inherent to the *P. esculentus* complex, where rare events due to untypical gametogenetic processes can lead to the raise, the perpetuation and the dispersion of new evolutionary significant lineages which may also deserve special conservation measures.

Introduction

Fertile taxa of hybrid origin are pushing the biological species concept to its limits (Dobzhansky 1937; Mayr 1942; Mallet 2008). By allowing genetic interactions between well defined and differentiated taxa, hybrids are challenging the most acknowledged mode of speciation by divergence followed by reproductive isolation, and they allow scrutinizing the consequences of gene transfer between "good species". Hence hybrids constitute biological models of high interest in evolutionary biology and represent valuable material for the ongoing debate on the definition of the nature of species (i.e. whether they are real entities or just arbitrary constructs of the human mind) and on the process of speciation (Mallet 2001; Coyne and Orr 2004; Abbott et al. 2008).

Secondary contact of diverged genetic entities can lead to hybridization when it happens before effective premating barriers have developed. However, failure in segregation of chromosomes from different species often leads to a tremendous fitness decrease in the hybrids' offspring, ranging from zygotic mortality to inviability or infertility. Some hybrid taxa have escaped the genetic incompatibilities and the resulting detrimental effects on fitness by abandoning normal meiosis. In vertebrates, they have shifted from sexual to clonal genome transmission and adopted one of the following three reproductive modes:

• In parthenogenesis, offspring develop from unreduced eggs without any male input.

• In gynogenesis such unreduced egg need the contact with sperm to trigger the development, but do not incorporate the paternal genetic material.

• In hybridogenesis (Schultz 1969), one of the parental genomes is excluded during the first steps of meiosis, followed by the production of clonal gametes containing the other parental genome. By living in sympatry and mating with the parental species, whose genome has been excluded, hybridity is re-established and thus a hemiclonal hybrid line perpetuated. Such a reproductive mode has been shown to exist and be quite stable in natural animal populations of insects (*Bacillus*, Mantovani and Scali 1992), fishes (*Squalius,* Carmona et al. 1997, and *Poeciliopsis,* Schultz 1966) and anurans (*Pelophylax*, Berger 1968).

Where problems of chromosome pairing during gametogenesis lead to occasional failure or regular circumvention of chromosome segregation, and hence the production of unreduced gametes, an increase of the ploidy level of the offspring

can result (Vrijenhoek 1989; Ramsey and Schemske 1998). Thus, there is a link between hybridization, asexual reproduction and polyploidisation which creates genetic systems with the potential for hybrid speciation through allo-polyploidisation (Choleva et al. 2012).

The probability of establishing an independently evolving polyploid hybrid lineage can be expected to increase with (1) the rate and type (in terms of genomic composition) at which unreduced gametes are produced, (2) the likelihood that they will fuse, (3) the viability and fertility of the resulting allopolyploid offspring, and (4) the reproductive isolation of such offspring from its parental species and their competitive ability. Chances of establishing a stable and self-perpetuating polyploid lineage are expected to be highest for even-ploidy (e.g. tetraploidization) because it allows biparental reproduction with normal meiosis. It has been shown, however, that triploid forms producing diploid gametes in one sex and haploid ones in the other sex can act as a stepping stone towards tetraploidization (triploid bridge; Ramsey and Schemske 1998; Mable 2004; Cunha et al. 2008). Moreover, as hybrids are often capable of occupying habitats beyond the limits of their diploid progenitors (Endler 1973; Moore 1977; Arnold 1997), we can expect that if such hybrids manage to produce the necessary gamete types, they can replace populations of their parental species. Thus, under certain genetic and ecological conditions hybrids can become evolutionary independent units.

The evolutionary impact of hybridization and polyploidy has been well demonstrated among plant species (Stebbins 1950; Grant 1971; Rieseberg 1997), but examples from the animal kingdom are scarce, especially when it comes to vertebrates (Arnold 1997; Mallet 2008; Schwenk et al. 2008). In this study, we address the first above mentioned condition for polyploidy, i.e. the types of gametes produced by different genomotypes, in anuran populations containing triploid individuals.

The Pelophylax esculentus complex

An excellent model system for investigating the evolutionary impact of polyploid hybrids and the associated shift from sexual to clonal genome transmissions is provided by Palearctic water frogs of the *Pelophylax esculentus* complex (formerly genus *Rana* until Frost 2006). The complex is composed of two parental species, the pool frog *P. lessonae* (Camerano, 1882) and the marsh frog *P. ridibundus* (Pallas, 1771), and their inter-specific hybrid *P. esculentus* (Linnaeus, 1758), the edible frog.

Hybrids of both sexes overcome meiotic pairing problems of *lessonae* (L) and *ridibundus* (R) chromosomes by excluding one of the parental genomes during the first division of gametogenesis and transmitting only the other genome (hybridogenesis; Schultz 1969; Graf and Müller 1979). The original hybrid status is restored by mating with a partner that provides the eliminated genome.

This basic pattern comes in three major variations. In the most widespread case, diploid hybrids (genotype LR) exclude the L genome, produce haploid gametes with a clonal R genome and restore hybridity by mating with *P. lessonae* (genotype LL). Thus, they are forced to live in sympatry with at least this parental species, thus constituting so-called LE-systems. In the mirror system, named RE-system, the R genome is excluded, and the L genome transmitted, which forces *P. esculentus* to live and mate with *P. ridibundus* (genotype RR) to perpetuate its hybrid line. There is a tendency for LE-systems to be more frequent in Western Europe and RE-systems to dominate in Eastern Europe, but numerous exceptions exist. What generates these two breeding systems remains a puzzle, because the exact mechanisms of genome exclusion are still not known; nor are the factors that determine which parental genome is inducing, respectively resisting, exclusions under what conditions. In both systems, however, the hybrids are acting as sexual parasites of a parental host species.

In the northern parts of the species' range, especially around the Baltic Sea, a third breeding system type exists: the EE-system (Plötner 2005; Christiansen 2009; Arioli et al. 2010; Jakob et. al. 2010). Here, populations consist of hybrids only, with no parental species occurring in the surrounding area. Those all-hybrid populations are composed of diploid hybrids (genome LR) and triploids with the LLR and/or LRR genome composition. In this system, diploid females usually produce diploid LR gametes, whereas triploids produce haploid gametes containing the recombined genome of the type they have in double dose, i.e. L in LLR frogs and R in LRR (Christiansen 2009; Christiansen and Reyer 2009). This mechanism has been termed "meiotic hybridogenesis" (Alves et al. 1998; Morishima et al. 2008). The production of these three gamete types allows the generation and persistence of the all-hybrid populations. Differences in gamete production, rather than variation in ecological selection regimes, seem to explain why the proportions of LR, LLR and LRR frogs differ among ponds (gamete pattern hypothesis versus selection hypothesis; Christiansen et al. 2010; Embrecht and Reyer 2012).

These findings are based on intensive studies of all-hybrid populations in Denmark and southern Sweden (Christiansen and Reyer 2009, Arioli et al. 2010, Jakob et. al. 2010). However, triploid hybrids have also been reported for several populations south of the Baltic Sea and in Central Europe, where they occur either with only diploid hybrids or with diploids and one or both parental species together (Berger 1988a; Tunner 1992; Mikulíček and Kotlik 2001; Plötner 2005).

So far, detailed water frog studies have focused on populations within a limited geographic area and on a particular system, i.e. either LE- and RE-systems where diploid hybrids live and mate with a parental species or EE-system where diploid and triploid hybrids co-occur in the absence of any parental species. However, given the marked regional differences among populations, we felt that a large-scale comparative study between populations with and without triploid individuals was needed. The purpose of our study was to systematically investigate regional and genotype differences in gamete production, their consequences for the breeding system, and whether triploid frogs are of mono- or polyphyletic origin. For this study, we sampled five European populations from four different river basins and performed two different analyses. First, we conducted crossing experiments to analyse the types of gametes produced by the different hybrid genomotypes, i.e. the genomic constitution in terms of the number and origin of the constitutive genomes (Lowcock 1994). Second, we used microsatellite analysis to calculate population genetics parameters, such as expected heterozygosity (H_e, Nei 1978) and fixation index, (F_{ST}, Weir and Cockerham 1984). Together, the two approaches allowed us to infer the breeding systems and their similarities, respectively difference, in different populations. Based on our results, we then discuss possible origins of the systems, the evolutionary potential they carry and their conservation value.

Material and methods

Populations

We sampled frogs in five populations from three European countries (Figure 1). In Poland, frogs were caught from two ponds located near Wysoka Kamieńska (53°49'18"N, 14°50'38"E, in this study referred to as Wysoka). In Germany, they originated from one pond situated 2 km south of the village of Herzberg am Harz (51°37'37"N, 10°21'15"E, Herzberg) , and from the village pond of Schönermark, near Kyritz (52°54'08"N, 12°19'16"E, Kyritz). In Slovakia we sampled from two ponds close to the village of Šajdíkove Humence (48°38'34"N, 17°16'54"E, Šajdíkove) and from two ponds located in the village of Šaštín-Stráže (48°37'55"N, 17°08'40"E, Šaštín). Maximum distances between the five populations were 580 km in north-south and 470 km in east –west direction.

Figure 1: Locations of sampled populations in Germany, Poland and Slovakia.

Frogs were collected by hand at night using a flashlight. They were identified for sex and taxon on the spot according to phenotypic characteristics (Berger 1988b; Plötner 2005). In order to distinguish diploid from triploid hybrids, we took blood smears and measured erythrocyte lengths and widths under the microscope; in *Pelophylax* triploid erythrocytes are significantly larger than diploid ones (Berger 1988a, Vinogradov 1990). All frogs were toe clipped for subsequent microsatellite DNA analyses in order to confirm the taxon identification and analyze genotype composition in the total sample. Thereafter, most frogs were released back into the pond of origin; but a few diploid and triploid hybrids were kept for crossing experiments in the lab. They were selected on the basis of their size, health and, in females, signs of gravity. These kept frogs were individually marked with transponders (ID-162, AEG), separated by sex and assumed genotype and transported to the University of Zurich in cloth bags filled with rubber sponges. During transport, the bags were showered daily with fresh water. All frogs survived the journey.

Microsatellite analysis

Precise genotype identification of the frogs sampled on site, of the frogs used as parents, and of the offspring resulting from the crosses, was achieved through microsatellite analysis. We used a piece of the tailfin (tadpoles) and a fingertip (adults and metamorphs), respectively, as source material. DNA extraction and purification were performed using a Biosprint 96 DNA Blood Kit (Qiagen) in combination with the Biosprint 96 workstation following the supplier's protocol. The purified DNA was subjected to PCR runs with four primer mixes involving a total of 18 microsatellites primer pairs:

• Primer Mix 1A: CA1b6, Ga1a19 redesigned (Arioli et al. 2010), RlCA1b5, RlCA5 (Garner et al. 2000), Rrid064A (Christiansen and Reyer 2009)

• Primer Mix 1B: Re2CAGA3 (Arioli et al. 2010), Res16, Res20 (Zeisset et al. 2000), RlCA2a34 (Christiansen and Reyer 2009)

• Primer Mix 2A: ReGA1a23, Rrid169A, Rrid059A redesigned (Christiansen and Reyer 2009), Res22 (Zeisset et al. 2000), Rrid013A (Hotz et al. 2001)

• Primer Mix 2B: Re1Caga10 (Arioli et al. 2010), RlCA18 (Garner et al. 2000), RlCA1a27, Rrid135A (Christiansen and Reyer 2009).

Details on PCR protocols are given by Christiansen (2009) and Christiansen and Reyer (2009, 2011). PCR products were run for fragment length analysis on an ABI

3730 Avant capillary sequencer with internal size standard (GeneScan-500 LIZ), and the alleles were scored with the Genemapper software v3.7 (Applied Biosystems).

Loci Res20, RlCA2a34, ReGa1a23, RlCA1a27 and RlCA18 and were species-specific for *P. lessonae* while loci Rrid064A, Re2CAGA3, Res22, Re1CAGA10 and Rrid135A were species-specific for *P. ridibundus*. The other eight microsatellite loci amplified in both L and R genomes. For these loci species-specificities of the alleles were known from previous studies (Christiansen 2005; Christiansen 2009; Arioli et al. 2010; N.B.M. Pruvost unpublished data). Four microsatellite loci (CA1b6, RlCA1b5, Ga1a19redesigned and Res16) showed a dosage effect allowing us to determine the ploidy of hybrids by comparing the height of the peaks (Christiansen 2005). The sum and congruence of the 18 microsatellites markers allowed the identification of the consensus genotype of each specimen.

Population genetics analyses

Because of the hybridogenetic mode of genome transmission which inhibits recombination between the *P. lessonae* (L) and *P. ridibundus* (R) genomes, all analyses were performed for each genome separately. Prior to analyses we tested the microsatellite dataset for the presence of null alleles in both genomes using the software Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004). Because the procedure implemented in Micro-Checker requires diploid data, we could apply this method only to the specimens of the two parental species and to triploid hybrids for the genome present in double copy. For haploid parental genomes, i.e. single-copy genomes of triploids and both genomes in diploids, the search for null alleles was done by simple examination of the data. When even after two to three re-runs of PCR, no allele was detected, this was taken as an indication for the presence of a null allele. Null alleles were detected in two loci that amplify for both genomes, namely RlCA5 and Res16. In addition, loci RlCA2a34, ReGA1a23, Rrid169A showed the presence of null alleles in the R genome, while locus Re1CAGA10 betrayed a null allele in the L genome. After excluding these loci from further analyses, we could use the following ten loci for our calculations: CA1b6, RlCA1b5, Ga1a19redesigned, Rrid013A and Rrid059redesigned for both genomes, together with Res20, RlCA2a34, ReGA1a23, RlCA1a27 and RlCA18 for the L genome only, and with Rrid064A, Re2CAGA3, Res22, Re1CAGA10 and Rrid135A for the R genome only.

We investigated population structure by calculating the allelic diversity corrected for sample size ($H_{e,}$ expected heterozygosity according to Nei 1978) and

the fixation index (F_{ST}, according to Weir and Cockerham 1984) using the software SPAGeDi version 1.3 (Hardy and Vekemans 2002) which allows the combination of multiple ploidy levels in the same analysis. Again, because of the independence of the two parental genomes, expected heterozygosity was calculated separately for the L genome (H_{eL}) and for the R genomes (H_{eR}) for each frog genomotype in each of the studied populations. In order to investigate, how similar, respectively different gene pools are, we compared allelic diversity values between pairs of gene pools of different frog types, by applying two-tailed paired t-tests to the values for each locus. We also run non-parametric Wilcoxon signed-rank test which gave the same results. For comparisons between more than two types of frogs within a population we used analyses of variance with H_e as dependant variable and loci as fixed effect.

In order to estimate the genetic distances between each genetic pool of different frog types in each population, we calculated pairwise F_{ST} values separately for the L genomes of the LL, LR, LLR and LRR frogs and for the R genomes of the LR, LLR, LRR and RR frogs, respectively. P values for these F_{ST} were obtained by running permutation test with 10 000 iterations. Concerning the interpretation of these values we followed the qualitative guideline proposed by Wright (1978): $0 \leq F_{ST} < 0.05$ indicate little genetic differentiation, $0.05 \leq F_{ST} < 0.15$ moderate, $0.15 \leq F_{ST} < 0.25$ great, and $0.25 \leq F_{ST}$ very great genetic differentiation.

All statistical tests were run using the program R (version 2.15.1, R Development Core Team 2012).

Crossing design

In order to determine the type of gamete produced by a given hybrid and to avoid the masking effect of potential genetic incompatibilities between hybrid genomes, we crossed each frog with at least one specimen of each parental species (*P. lessonae* and *P. ridibundus*) and with one other hybrid.

We originally had planned to cross three hybrids of each genomotype from the five populations but due to insufficient egg numbers in some females and/or failed fertilization through sperm of some males we could not systematically do this (see Table 1). For the same lack of gametes, we also did not perform crosses between parental males and females; but parental offspring resulting from such combinations are not relevant for our questions anyway.

Table 1: Population composition, in term of number of frogs caught and number of frogs crossed per genomotypes, for two mixed population (M) where diploid hybrids occur in sympatry with a parental species and three all-hybrid populations consisting of diploid and triploid hybrids. "-" stands for the absence of frogs of the respective type, "x" stands for frog types which were present in the population but not crossed. Some of the parental species specimens used in crosses came from other populations and are not listed here.

Population		Genomotype									
		LLR		LR		LRR		LL		RR	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Herzberg	Caught	-	-	6	19	-	-	-	10	-	25
(M)	Crossed	-	-	3	3	-	-	-	X	-	X
Šaštin	Caught	-	-	43	27	-	-	1	27	13	15
(M)	Crossed	-	-	5	5	-	-	x	4	2	3
Šajdíkove	Caught	-	91	30	2	-	-	-	-	-	-
(H)	Crossed	-	14	5	1	-	-	-	-	-	-
Kyritz	Caught	7	19	34	25	24	12	-	1	-	-
(H)	Crossed	2	3	3	3	3	3	-	x	-	-
Wysoka	Caught	3	14	17	10	7	6	-	-	-	-
(H)	Crossed	X	2	2	5	1	1	-	-	-	-

Artificial crossing procedure

Crosses were performed following the artificial fertilization procedure by Berger et al. (1994) with minor modifications. Ovulation stimulation was triggered by the injection of a solution of LHRH fish hormone (Bachem H-7525) at 2 mg in 100 ml Holtfreter's solution. We injected 100µl per 10 g of body mass. After about 24 hours, when females were ready for laying eggs, males were euthanized in a buffered (pH 7) MS-222 solution (Sigma A-5040) at 2mg/l and their testes were removed, sliced and crushed in a Petri dish with aged tap water. Eggs were gently stripped into this sperm suspension, where they remained for about 2-3 minutes. After this period, the suspension was rinsed into a new Petri dish where eggs of another female were added. This protocol allows the use of the same sperm solution to fertilize eggs from different females and to fertilize eggs of the same female with sperm from different males. Eggs were covered with aged tap water and checked for fertilization success, identified by a rotation that moves the black animal hemisphere to the top within the next 30-60 min. The next day, all eggs were transferred to 6 l containers with 1-2 cm of water. After two days unfertilized eggs, egg jelly and/or aborted embryos were carefully removed every two days to avoid bacterial and fungal development. After

about 15 days embryos started to reach free swimming stage (stage 25, Gosner 1960) and were euthanized using the MS-222 buffered solution cited above. The offspring of a few crosses were used for other experiments (Pruvost et al. 2013) but their genotypic data could also be use for our purpose. All studied offspring reached at least stage 25.

Gamete production determination

Originally, we had planned to genotype a minimum of 35 offspring for each cross. However, due to limited egg availability, low fertilization success and/or inviable offspring, probably resulting from genetic incompatibilities, for some crosses this goal was not reached, while for others more than 35 offspring could be genotyped (see Appendix 1). After identifying the offspring genotypes, and knowing the genotypes of their mothers and fathers, we could determine the types and relative numbers of gametes produced by each of the two parents. Since each parent frog was used in more than one cross, we summed up the results obtained from all crosses involving this frog. Potential problems caused by parental infertility or genetic incompatibilities which may mask the actual gamete production would have been revealed by a differential gamete production patterns among crosses involving the same frog. If, for instance, a frog produced no viable offspring with any of the individuals it was crossed to, this would indicate infertility, whereas failure in only one or the other cross suggests genetic incompatibility with the particular partner. However, neither was found.

Results

Population composition

Microsatellite analysis allowed us to determine the genomotypes of 488 adult frogs sampled in the five populations. Population compositions in terms of taxa and ploidy are shown in Table 1. In two populations (Herzberg, Šaštin) – from now on called “mixed populations” – diploid hybrid males and females occurred in sympatry with both parental species, whereas the other three populations only hybrids were found (“all-hybrid populations”), with the exception of one LL individual in Kyritz. In Šaštin, individuals of the two parental species existed in both sexes, but in Herzberg only males were captured.

The three all-hybrid populations also differed in their composition. In Kyritz and Wysoka, we caught all three possible genomotypes (LR, LLR, and LRR) in both sexes, but in Šajdíkove LRR was absent, LLR consisted exclusively of males and LR almost only of females (with the exception of two diploid males). Given the large number of frogs caught in this population (n=123, Table 1), this genotype and sex bias is highly unlikely to have resulted from chance effects in a small sample. In Šajdíkove microsatellite dosage effect revealed the presence of one tetraploid male (LLRR) possessing the same double L genome as the triploids in addition to a double R genome completely homozygote for the studied loci.

Populations genetic structure

Allelic diversity

The mean allelic diversity for the ten loci considered is shown in Table 2 for each genome separately and detailed by loci in Appendix 2. In the two mixed populations, L genome diversity (H_{eL}) did not differ between LR hybrids and parental LL (Šaštin: mean difference=0.007 ±0.032, $t_{(9)}$=0.215, p=0.834; Herzberg: m.d.=0.073 ±0.070, $t_{(9)}$=1.045, p=0.323)), nor did R genome diversity (H_{eR}) differ between LR and parental RR in Herzberg (m.d.=0.015 ±0.043, $t_{(9)}$=0.347, p=0.736); but in Šaštin it did (m.d.=0.240 ±0.050, $t_{(9)}$=4.799, p=0.001), with the *P. ridibundus* parental species showing a higher allelic diversity (H_{eR}=0.625) than the LR hybrids (H_{eR}=0.384).

With respect to the all-hybrid populations, analyses of variance did not detect any differences in both H_{eL} and H_{eR} between diploid (LR) and triploid (LLR, LRR) hybrids in Wysoka and Kyritz where all three genomotypes occur (Table 2). In contrast, in Šajdíkove, with (mostly) LR females and only LLR males, H_{eL} values differ greatly between diploids and triploids (m.d.=0.251 ±0.080, $t_{(9)}$=3.130, p=0.012) with diploid hybrids showing a higher allelic diversity than the triploid LLR males. While H_{eR} values do not (m.d.=0.029 ±0.016, $t_{(9)}$=-1.862, p=0.095). In this population the allelic composition of all expressed loci of the double L genome of the triploid males is exactly the same among all specimens, meaning that all LL genomes in all LLR males are genetically identical.

Table 2: Mean allelic diversity corrected for sample size, Nei 1978 (H_e) for *P. lessonae* genomes (H_{eL}) and *P. ridibundus* genomes (H_{eR}) in the different frog types (LL, LLR, LR, LRR and RR). Sample size is given in brackets.

Pop.	H_{eL}				H_{eR}			
	LL	LLR	LR	LRR	LLR	LR	LRR	RR
Herzberg	**0.441** (10)	-	**0.368** (25)	-	-	**0.380** (25)	-	**0.395** (25)
Šaštin	**0.428** (28)	-	**0.421** (70)	-	-	**0.384** (70)	-	**0.625** (28)
Šajdíkove	-	**0.201** (91)	**0.452** (32)	-	**0.432** (91)	**0.402** (32)	-	-
Kyritz	-	**0.321** (26)	**0.300** (59)	**0.284** (36)	**0.358** (26)	**0.404** (59)	**0.401** (36)	-
Wysoka	-	**0.240** (17)	**0.221** (27)	**0.212** (13)	**0.512** (17)	**0.554** (27)	**0.609** (13)	-

Population differentiation

The overall genetic differentiations (represented by global F_{ST} values) shows substantial and highly significant differentiation among populations for both genomes, assigning 43.59% of the variation in the L genome (global F_{ST}=0.436, $p<0.001$) and 25.42% in the R genome (global F_{ST}=0.254, $p<0.001$) to inter-population differences.

The pairwise F_{ST} values between each frog genomotype in each population are given in Table 3. In the two mixed populations, there is little differentiation between LR and LL in the L genome (Šaštin: F_{ST}=0.028; Herzberg: F_{ST}=0.024) and little to moderate differentiation between LR and RR in the R genome (Herzberg: F_{ST}=0.033; Sastin: F_{ST}=0.138). Among the all-hybrid populations, differentiation is low for both genomes within Wysoka and Kyritz, where all three hybrid types occur (all $F_{ST} \leq 0.041$) In Šajdíkove, with only two hybrid types differentiation between LLR males and mostly LR females is also low for the R genomes (F_{ST}=0.008), but very high for the L genomes (F_{ST}=0.517).

Table 3: Pairwise F_{ST} values using Weir and Cockerham calculation (1984). Values for the R genomes are above the diagonal and values for the L genomes under it. $0 \leq F_{ST} < 0.05$ indicate little genetic differentiation (uncolored boxes), $0.05 \leq F_{ST} < 0.15$ moderate (light green for L and light orange for R), $0.15 \leq F_{ST} < 0.25$ great (green for L and orange for R), $0.25 \leq F_{ST}$ very great genetic differentiation (dark green for L and dark orange for R) (Wright, 1978).

L\R	HerLL	HerLR	HerRR	KyrLLR	KyrLR	KyrLRR	SajLLR	SajLR	SasLL	SasLR	SasRR	WysLLR	WysLR	WysLRR
HerLL	x	-	-	-	-	-	-	-	-	-	-	-	-	-
HerLR	0.024	x	0.033	0.347	0.358	0.363	0.331	0.374	-	0.394	0.260	0.296	0.260	0.287
HerRR	-	-	x	0.378	0.375	0.379	0.314	0.362	-	0.392	0.274	0.333	0.294	0.313
KyrLLR	0.437	0.444	-	x	0.041	0.036	0.298	0.330	-	0.322	0.241	0.247	0.235	0.251
KyrLR	0.464	0.470	-	0.016	x	0.000	0.311	0.344	-	0.335	0.250	0.249	0.233	0.237
KyrLRR	0.461	0.470	-	0.040	0.017	x	0.300	0.327	-	0.326	0.255	0.240	0.227	0.228
SajLLR	0.634	0.655	-	0.616	0.625	0.643	x	0.008	-	0.095	0.148	0.213	0.207	0.213
SajLR	0.353	0.353	-	0.275	0.311	0.311	0.517	x	-	0.062	0.155	0.225	0.209	0.218
SasLL	0.371	0.374	-	0.213	0.233	0.213	0.522	0.091	x	-	-	-	-	-
SasLR	0.352	0.351	-	0.215	0.239	0.224	0.506	0.087	0.028	x	0.138	0.276	0.262	0.271
SasRR	-	-	-	-	-	-	-	-	-	-	x	0.148	0.144	0.125
WysLLR	0.499	0.501	-	0.167	0.193	0.210	0.667	0.361	0.272	0.293	-	x	0.000	0.000
WysLR	0.520	0.525	-	0.138	0.170	0.189	0.669	0.361	0.267	0.288	-	0.003	x	0.000
WysLRR	0.464	0.494	-	0.109	0.152	0.179	0.683	0.319	0.244	0.267	-	0.000	0.000	x

Gamete production

We performed a total of 198 crosses involving 64 *P. esculentus* (35 LR, 21 LLR, and 8 LRR), 18 *P. lessonae* and 15 *P. ridibundus*. We genotyped the 97 adults crossed and 4´675 tadpoles resulting from these crosses. The results of the gametes produced are presented in Appendix 1.

In two populations we encountered problems which resulted in low offspring numbers or even no offspring at all (for details see column N off. in Appendix 1). These problems resulted from lack of sufficient mature eggs in some females, sexual immaturity of few males and a combination of the two causes. Overall, however, we managed to analyse the proportions of gamete types produced by every hybrid type in each population, except for the only LLR males from Wysoka (see Appendix 1). In the mixed populations of Herzberg and Šaštin, hybrid LR frogs of both sexes always produced haploid gametes with a clonally transmitted R genome. Among the all-hybrid populations, the pattern was more diverse.

In Kyritz, as well as in Wysoka, diploid males also exclusively produced haploid gametes with a clonally transmitted R genome, but all diploid females produced diploid LR gametes, with the exception of one female from Kyritz (WFB014-

20) which produced equal numbers of R and LR eggs. Among the triploids, the prevailing pattern was the production of haploid gametes with a recombined genome of the type that is present in two copies, i.e. L in LLR and R in LRR. Without any exception this was true for all LRR of both sexes and all LLR males, whereas in LLR females it applied to only 89% of the eggs. The remaining 11% contained diploid clonally transmitted LL genomes.

In Šajdíkove triploid males always produced diploid gametes, which clonally transmit two L genomes. The microsatellite genotyping revealed that the LL multilocus genotype of all these frogs is exactly the same in all adults males caught on site and in all the offspring produced by our crosses. The diploid males and females from this population produced only clonal haploid R gametes. The general pattern of gamete production is given in Table 4.

Table 4: Gamete production of the different genomotypes of hybrids and inferred breeding systems in the five studied populations. Gamete types in parentheses are produced in small proportions.

Population	Genomotype						Inferred breeding system
	LLR		LR		LRR		
	Female	Male	Female	Male	Female	Male	
Herzberg	**-**	**-**	**R**	**R**	**-**	**-**	L-E
Šaštin	**-**	**-**	**R**	**R**	**-**	**-**	L-E
Šajdíkove	**-**	**LL**	**R**	**R**	**-**	**-**	Modified L-E
Kyritz	**L** (LL)	**L**	**LR** (R)	**R**	**R**	**R**	E-E
Wysoka	**L**	**L**	**LR**	**R**	**R**	**R**	E-E

Discussion

The gamete production patterns found in this study confirm the expected mixture of clonally and recombining genomes travelling between different frog genomotypes. In combination with H_e and pairwise F_{ST} values, which allow estimating levels of genetic differentiation between gene pools of all frog genomotypes, we can describe the genetic interactions happening in the different populations and link them to known breeding system types occurring in water frogs. In the following paragraphs, we propose an evolutionary scenario for the appearance and maintenance of these systems.

Gamete production pattern

Diploid hybrids always transmitted clonal genomes, either haploid R or diploid LR. The production of haploid gametes with clonal R genomes is in accordance with the hemiclonal transmission mode expected in LE-systems (Figure 2), where the previously excluded L genome is regained by mating with *P. lessonae*, and thus hybridity restored. In contrast, the production of diploid gametes carrying clonal copies of the entire LR maternal genome is a feature expected of diploid females from all-hybrid populations of the EE-system (Figure 4) (Christiansen 2009). Here, the L and R genomes that are necessary for maintaining all three hybrid types in the population (LR, LLR, LRR) are provided by triploids that produce recombined haploid gametes of the type that is present in two copies (Christiansen and Reyer 2009; Morishima et al. 2008). With the slight modification in two Kyritz LLR females which produced a few diploid gametes containing their two L genomes, this was the pattern found in triploid frogs from Kyritz and Wysoka.

While these results confirm those from previous studies, the gamete production pattern in LLR males from Šajdíkove, with clonally produced sperm containing their double L genomes, suggests a previously not described "modified LE-system" (Figure 3). Below, we discuss the three breeding systems in more detail.

Breeding systems

LE-system (Figure 2)

In typical LE-systems, diploid hybrids discard the L genome prior to meiosis, produce clonal R gametes and restore hybridity by mating with the sexual *P. lessonae* parental species which provides gametes with a new recombined L genome. In such

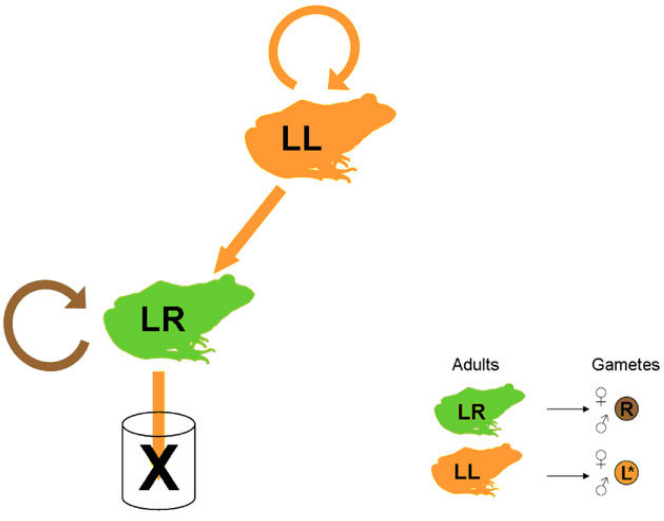

Figure 2: "LE-system" scheme showing the transmission of the L (orange arrow) and of the R (brown arrow) genomes and the gamete production pattern of the different frogs genomotypes. The * in the gametes indicates recombining genomes.

systems, the hybrids are sexual parasites of the *P. lessonae* parental species and act as a sink for the host's L genome (Schmidt 1993; Joly 2001; Lehtonen et al. 2013). In our study, this system is represented by the populations in Herzberg and Šaštin.
In Šaštin, allelic diversity in the R genome (H_{eR}) is lower in LR hybrids (with no recombination) than in RR frogs (with recombination), and there is moderate genetic differentiation between LR and RR frogs (F_{ST} = 0.138). In contrast, allelic diversity in the L genome (H_{eL}) is equally high for LR and LL frogs and genetic differentiation between their genomes is low (F_{ST} = 0.028) (Table 2). This is in line with the genome transmission mode in LE-systems: clonal R versus sexual L.
In Herzberg the situation appears a bit different regarding the role of the sympatric *P. ridibundus* frogs. The relatively low genetic differentiation between LR and RR frogs in the R genome and the quite similar values of gene diversity are a hint close interactions between the two gene pools. In both populations, however, allelic diversity and genetic differences may not only reflect the genome transmission mode but also be influenced by the number of original primary hybridization which will affect diversity in the clonal R genome. Unfortunately, empirical data about primary hybridizations are lacking for both populations.

Modified LE-system (Figure 3)

In Šajdíkove the gamete production pattern of the diploid hybrids is the same as the one occurring in LE-systems, but this population also contains triploid hybrid LLR males, which always produce diploid LL gametes containing identical copies of the two same genomes. This mode of transmission is clearly reflected by the population genetic indices:

• First, the F_{ST} value estimating the differentiation of the L genome between LLR and LR frogs within Šajdíkove is very high.

• Second, allelic diversity in the L genomes is significantly lower in LLR frogs (H_{eL}=0.201) which receive a clonal LL genome than in LR frogs (H_{eL}=0.452), where the value is similar to those of LL and LR frogs from LE-systems (Table 2). This suggests that diploid hybrids in Šajdíkove received recombined L genomes. Another, not mutually exclusive, explanation for the higher allelic diversity in L genomes of LR frogs is that new lineages have been produced on multiple occasions.

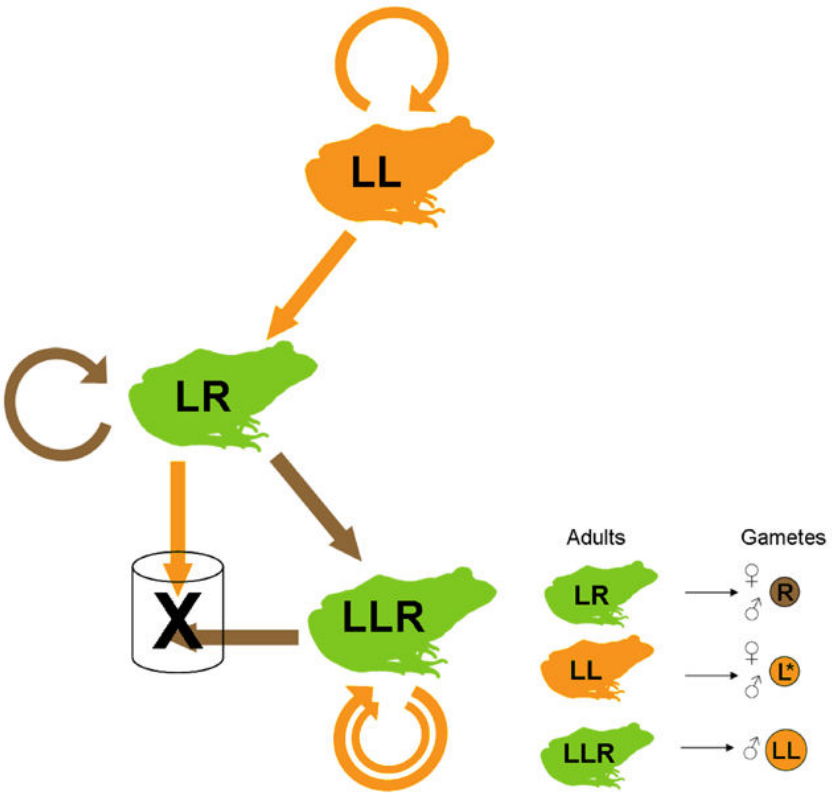

Figure 3: "Modified LE-system" scheme showing the transmission of the L (orange arrow) and of the R (brown arrow) genomes and the gamete production pattern of the different frog genomotypes. The * in the gametes indicates recombining genomes.

However, both explanations cannot answer the question where the haploid L genomes (which are required to produce diploid hybrids) originate from. In the sampled ponds, no *P. lessonae* were found. They may occur in ponds nearby. This hypothesis is consistent with the moderate genetic differentiation values found between the diploid LR from Šajdíkove and both the diploid LR and parental species LL in Šaštin. Also, haploid L gametes may occasionally be produced by diploid LR (as in the RE-system) or by triploid LLR (as in most EE-systems). For this, however, our crossing experiment provided no evidence (see Appendix 1). The triploid males that transmit their double L genome and mate with diploid LR females producing R eggs sire offspring of their own genomotype. Hence, they form a unique paternal hemiclonal lineage with a frozen L genome. Since these LLR frogs exclude the R genome at gametogenesis, they are acting as a sink for the R genome, which is transmitted by LR frogs that, in turn, are acting as a genetic sink for the L genome (Figure 3). Given that the L genome of the diploids must come from another source (see above), the triploid males in the population are not essential to the perpetuation of the diploids in the breeding system. They just seem to have found a way to persist by parasitizing the R genomes of the sympatric LR hybrids. In contrast to EE-

systems, which could not exist without triploids (see below), LLR males in Šajdíkove can be seen as a mere add-on to the L-E system. We, therefore, decided to name such breeding system type is not restricted to this western Slovak population. Some triploid LLR males carrying the same two genomes (with only a 2 bp difference in one allele out of the 18 microsatellite loci) have also been found in populations from the north-eastern part of the Czech Republic, 130 km north, in the locality of Borovec (Pruvost et al. in prep.).

EE-systems (Figure 4)

The gamete production pattern of frogs from Kyritz and Wysoka corresponds to the EE-system that was intensively studied and described for Denmark and southern Sweden by Christiansen and Reyer (2009), Arioli et al. (2010) and Jakob et al. (2010). In such systems, the three different hybrid genomotypes manage to produce all the gamete types needed for their coexistence without requiring the presence of any of the two parental species. Diploid LR eggs are produced by diploid LR females, haploid R sperm by diploid LR males, and recombined haploid L and R gametes by males and females of triploid LLR and LRR, respectively. This genetic functioning is perfectly reflected in the two population genetics parameters we used. In both populations, the gene diversity values for both genomes are in the same range for the three frog genomotypes. Pairwise F_{ST} values within populations also demonstrate very little genetic differentiation between the three genomotypes. In such breeding systems all frog genomotypes depend on each other to be produced (Figure 4):

• LR frogs arise from the combination of L gametes, exclusively produced by LLR frogs, with R gametes produced by LRR specimens, LR males and (in smaller proportion) LR females.

• LLR frogs mainly arise from fertilization of LR eggs produced by LR females with L sperm from males of their own genomotype, or (in smaller proportions) by fusion of R sperm coming from LRR and LR males with LL eggs from females of their own genomotype.

• LRR frogs only arise from the combination of LR eggs from LR females and R sperm produced by LR and LRR males.

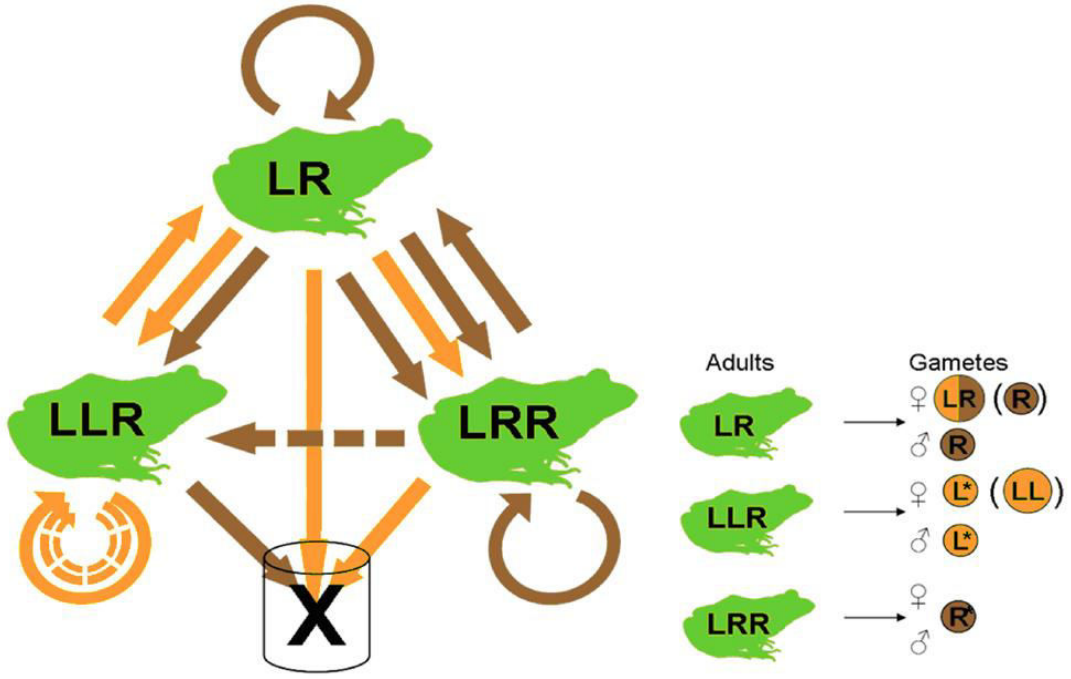

Figure 4: "EE-system" scheme showing the transmission of the L (orange arrow) and of the R (brown arrow) genomes and the gamete production pattern of the different frog genomotypes. Gamete types in parenthesis are produce in low frequency. Dashed arrows represent transmission with low frequency. The * in the gametes indicates recombining genomes.

Thus, LR and LLR frog types are absolutely necessary to the system in their role as producers of LR and L gametes, respectively, whereas LRR frogs are crucial as producers of R gametes, especially R eggs which only rarely are produced by LR females. Under these conditions, the EE-system would collapse if one of the actors would be removed. As predicted by the model of Som and Reyer (2006), such EE-system can persist under random mating which, indeed, seems to occur. In contrast to hybrid females from LE systems that prefer *P. lessonae* over *P. esculentus* males (Abt and Reyer 1993; Roesli and Reyer 2000; Engeler and Reyer 2001), females from all-hybrid populations show no preference (Günther and Plötner 1989; Rondinelli 2006). Since triploid hybrids recombine the genome they have in double dose (Christiansen and Reyer 2009), they provide genetic diversity equivalent to the one found in the parental species, giving such systems an evolutionary potential comparable to that of sexually reproducing populations.

Origins and evolutionary potential of systems involving triploid hybrids

The difference in gamete production patterns, leading to the existence of triploid specimens in Wysoka and Kyritz on the one hand and in Šajdíkove on the other

strongly suggests a polyphyletic origin of triploid frogs in EE- and modified LE-systems. Both systems may have developed from the most wide-spread typical LE-system (Figure 2), because all three systems are identical in that LR males produce clonal haploid R gametes; but then differences arose from the mechanisms that lead to the production triploid individuals: fusion of LR eggs from LR females with haploid sperm in the EE-system as opposed to fusion of haploid eggs with LL sperm from LLR males in the modified LE-system. The perfect identity of the two L genomes present in triploid LLR males from the modified LE-system suggests that this lineage probably arose from a single event of L genome doubling that generated an array of clones, or even from one single triploid specimen. Unraveling the origin of such frogs would demand a much broader population genetics investigations. However, whatever their origin, the 3n males in this system do not participate in the generation of the two other frog types (LL and LR). They only exploit R genomes from the pool of eggs produced by LR females and use their own double L genome to procreate themselves. They act as a sink for the R genome which already parasitizes the parental species sexual L genome. Thus, in contrast to EE-systems which depend on the presence of triploids, triploids from the modified LE-system could disappear without harming the persistence of the other frog types, thus leaving an intact LE-system behind.

Concerning the EE-systems, the initial step away from the typical LE-system must have been a suppression of L genome exclusion in LR females, resulting in the clonal transmission of LR, rather than R genomes. Once produced, these 2n eggs automatically lead to both types of triploids: mating with *P. lessonae* males produces LLR offspring and mating with diploid *P. esculentus* hybrids produces LRR offspring. Due to the so-called meiotic hybridogenesis mechanism (Alves et al. 1998; Cuhna et al. 2008), LLR frogs are then able to produce recombined haploid L gametes and thus replace *P. lessonae* frogs, while LRR frogs can act as haploid R gamete donors and – in case of females – adopt the role previously fulfilled by LR females which now produce diploid LR eggs.

With the *P. lessonae* parental species having lost its essential position in maintaining the system, the hybrids become independent from the parental species, can disperse into environments where *P. lessonae* is absent and establish all-hybrid populations (EE-system). In combination with differential ecological tolerance leading to a competitive advantage for the hybrid these populations can be maintained, even

if later on the parental species also disperses into that habitat. In fact, the better performance of hybrids compared to the parental species under cold conditions, offers a possible explanation why the EE-system is wide-spread in colder region like the north of Europe (Negovetic et al. 2001; Pruvost et al. 2013),

This scenario highlights the high evolutionary potential of this seemingly flawed water frog system. What at first glance appears to be a failure of the typical gamete production pattern can, in situations where its meets favorable ecological condition, lead to completely new and evolutionary significant population types and breeding systems capable of colonizing new geographical ranges. Natural events and/or introduction may have led to some more population types and breeding systems with unusual combinations of different gametes donor types. Therefore, further detailed studies of the European water frog group seem justified and promising. Nevertheless, at least in the case of the EE-systems, our results support Schultz' (1989) statement "…non-Mendelian forms of hybrid origin have evolved adaptations distinct from parental biotypes and have assumed evolutionary directions that are different and independent of them".

This insight is also relevant from a conservation point of view. Modern management concepts stress the importance of conserving "evolutionary significant units" (ESUs), i.e. populations representing significant adaptive variation; but how these units are to be identified, is strongly debated (reviewed by Crandall et al. 2000; Pearman 2001). Hybrids, for instance, are exempt from protection, because they do not seem to constitute independent evolutionary lineages (Kraus 1995). This, however, does not hold for parthenogenetic, gynogenetic and hybridogenetic taxa that are originally of hybrid origin, propagate only the maternally inherited genome and may carry the potential for speciation via polyploidy. Depending on their genetic distinctiveness, their success in various environments and the effective size of their populations they, therefore, may require special protection efforts (Kraus 1995).

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Appendix 1: Gamete production of the crossed frogs. “Population” stands for the name of the population of origin, “Geno.” for the genotype of the parent, “Ind. Numb.” for its specimen number, “N cross” for the number of crosses involving this frog, “N off.” for the number of offspring genotyped and “Gamete type for the genomic composition and ploidy of the gametes produced.

Population	Geno.	Sex	Ind. numb.	N cross	N off.	Gamete type		
Herzberg	LR	F	WFB021-27	3	99	100 R		
			WFB021-28	4	103	100 R		
			WFB021-29	3	108	100 R		
		M	WFB021-12	5	73	100 R		
			WFB021-21	3	40	100 R		
			WFB021-22	3	57	100 R		
Kyritz	LLR	F	WFB014-21	3	74		91.9 L	8.1 LL
			WFB014-62	11	356		86.2 L	13.8 LL
		M	WFB014-55	4	173		100 L	
			WFB014-56	6	208		100 L	
			WFB014-59	7	143		100 L	
	LR	F	WFB014-20	7	12	50 R	50 LR	
			WFB014-25	10	468		100 LR	
			WFB014-63	7	158		100 LR	
		M	WFB014-05	7	303	100 R		
			WFB014-14	4	79	100 R		
			WFB014-48	6	156	100 R		
	LRR	F	WFB014-24	9	284	100 R		
			WFB014-26	7	272	100 R		
			WFB014-67	7	264	100 R		
		M	WFB014-11	6	161	100 R		
			WFB014-49	7	274	100 R		
			WFB014-58	4	143	100 R		
Šajdíkove	LLR	M	WFB007-93	4	86			100 LL
			WFB008-14	3	93			100 LL
			WFB015-13	4	10			100 LL
			WFB015-55	5	178			100 LL
			WFB015-56	2	11			100 LL
			WFB015-57	2	25			100 LL
			WFB021-16	3	3			100 LL
			WFB021-17	3	21			100 LL
			WFB021-18	3	16			100 LL
			WFB008-16	2	0			
			WFB015-09	6	0			
			WFB015-10	4	0			
			WFB021-19	2	0			
			WFB016-42	7	0			
	LR	F	WFB021-24	5	104	100 R		
			WFB021-30	3	96	100 R		
			WFB007-91	2	30	100 R		
			WFB021-25	1	0			
			WFB021-26	1	0			
		M	WFB007-90	2	8	100 R		
Šaštin	LR	F	WFB007-33	1	8	100 R		
			WFB007-35	1	12	100 R		
			WFB007-37	4	141	100 R		
			WFB015-72	8	283	100 R		
			WFB015-73	7	161	100 R		
		M	WFB007-52	4	101	100 R		
			WFB007-54	5	79	100 R		
			WFB015-03	6	84	100 R		
			WFB015-04	4	133	100 R		
			WFB015-06	7	254	100 R		

Wysoka	LLR	M	WFB003-02	2	66		100 L	
			WFB003-04	1	29		100 L	
	LR	F	WFB002-80	4	22			100 LR
			WFB002-81	2	45			100 LR
		M	WFB002-88	2	64	100 R		
			WFB002-92	2	66	100 R		
			WFB002-93	2	3	100 R		
			WFB002-94	1	1	100 R		
			WFB003-06	1	0			
	LRR	F	WFB002-74	4	6	100 R		
		M	WFB002-91	1	0			

Appendix 2: Allelic diversity corrected by sample size (Nei 1978) for each locus in the different frog types, for the L and the R genome respectively.

Population			All	Herzberg			Kyritz			Sajdikove			Sastin		Wysoka		
Genomotype			All	LL	LR	RR	LLR	LR	LRR	LLR	LR	LL	LR	RR	LLR	LR	LRR
N			434 L 449 R	10	25	25	26	59	36	91	32	28	70	28	17	27	13
L genome	Locus	CA1b6	0.415	0.479	0.513	-	0	0	0	0.503	0.498	0.486	0.448	-	0	0	0
		RICA1b5	0.136	0.337	0.347	-	0	0	0	0	0.446	0.308	0.248	-	0	0	0
		Ga1a19red	0.084	0.505	0.52	-	0	0	0	0	0	0	0	-	0	0	0
		Res20	0.685	0.668	0.417	-	0.652	0.521	0.348	0	0.665	0.203	0.472	-	0	0	0
		RICA2a34	0.838	0.468	0.587	-	0.741	0.831	0.833	0.503	0.665	0.785	0.755	-	0.212	0.268	0
		ReGa1a23	0.861	0.747	0.72	-	0.835	0.842	0.746	0.503	0.843	0.868	0.851	-	0.711	0.568	0.5
		Rrid013A	0.537	0.633	0	-	0.266	0.303	0.246	0	0.121	0.405	0.38	-	0.148	0.268	0
		Rrid059Ared	0.034	0	0	-	0	0	0	0	0	0	0	-	0.369	0.268	0.5
		RICa1A27	0.772	0	0.08	-	0.713	0.505	0.61	0	0.623	0.597	0.478	-	0.649	0.69	0.833
		RICA18	0.658	0.568	0.493	-	0	0	0.056	0.503	0.663	0.634	0.581	-	0.308	0.143	0.282
	Mean		0.502	0.441	0.368	-	0.321	0.3	0.284	0.201	0.452	0.428	0.421	-	0.24	0.221	0.212
	Standard Error		0.101	0.082	0.081	-	0.117	0.111	0.105	0.082	0.096	0.096	0.089	-	0.085	0.078	0.096
R genome	Locus	CA1b6	0.685	-	0.453	0.497	0.492	0.439	0.453	0.608	0.502		0.665	0.795	0.331	0.336	0.563
		RICA1b5	0.237	-	0.22	0.04	0.271	0.402	0.351	0	0	-	0	0.346	0.485	0.484	0.492
		Ga1a19red	0.5	-	0.28	0.078	0.077	0.345	0.263	0.452	0.353	-	0.162	0.638	0.471	0.647	0.668
		Rrid064A	0.644	-	0.513	0.509	0.271	0.129	0.108	0.602	0.554	-	0.111	0.349	0.603	0.711	0.75
		Re2CAGA3	0.88	-	0.663	0.691	0.754	0.712	0.691	0.788	0.805	-	0.712	0.768	0.757	0.852	0.855
		Res22	0.545	-	0.28	0.393	0.077	0.23	0.309	0.51	0.444	-	0.487	0.811	0.5	0.49	0.607
		Rrid013A	0.077	-	0.28	0.458	0	0	0	0	0	-	0	0.28	0	0	0
		Rrid059Ared	0.475	-	0.347	0.509	0.409	0.471	0.477	0.022	0.063	-	0.412	0.8	0.539	0.563	0.607
		Re1CAGA10	0.825	-	0.38	0.274	0.745	0.723	0.714	0.691	0.655	-	0.576	0.791	0.875	0.899	0.865
		Rrid135A	0.726	-	0.38	0.497	0.48	0.588	0.643	0.644	0.647	-	0.716	0.67	0.564	0.563	0.679
	Mean		0.559	-	0.38	0.395	0.358	0.404	0.401	0.432	0.402	-	0.384	0.625	0.512	0.554	0.609
	Standard Error		0.08	-	0.042	0.065	0.085	0.075	0.076	0.097	0.092	-	0.092	0.068	0.075	0.082	0.078

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Genomic effects on advertisement call structure in diploid and triploid hybrid waterfrogs (Anura, *Pelophylax esculentus*)

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Abstract

Background: In anurans, differences in male mating calls have intensively been studied with respect to taxonomic classification, phylogeographic comparisons among different populations and sexual selection. Although overall successful, there is often much unexplained variation in these studies. Potential causes for such variation include differences among genotypes and breeding systems, as well as differences between populations. We investigated how these three factors affect call properties in male water frogs of *Pelophylax lessonae* (genotype LL), *P. ridibundus* (RR) and their interspecific hybrid *P. esculentus* which comes in diploid (LR) and triploid types (LLR, LRR).

Results: We investigated five call parameters that all showed a genomic dosage effect, i.e. they either decreased or increased with the L/R ratio in the order LL-LLR-LR-LRR-RR. Not all parameters differentiated equally well between the five genotypes, but combined they provided a good separation. Two of the five call parameters were also affected by the breeding system. Calls of diploid LR males varied, depending on whether these males mated with one or both of the parental species (diploid systems) or triploid hybrids (mixed ploidy systems). With the exception of the northernmost mixed-ploidy population, call differences were not related to the geographic location of the population and they were not correlated with genetic distances in the R and L genomes.

Conclusions: We found an influence of all three tested factors on call parameters, with the effect size decreasing from genotype through breeding system to geographic location of the population. Overall, results were in line with predictions from a dosage effect in L/R ratios, but in three call parameters all three hybrid types were more similar to one or the other parental species. Also calls of diploid hybrids varied between breeding systems in agreement with the sexual host required for successful reproduction. The lack of hybrid call differences in a mixed-ploidy population at the northern edge of the water frog distribution is likely to be associated with genetic particularities, including a) low genetic variability and/or b) a local loss of genes coding for genotype-dependent call differentiation under conditions where female discrimination between diploid and triploid males is not beneficial.

Background

Acoustic communication in animals often mirrors selective forces that generate and maintain evolutionary change. In anurans, bioacoustic characteristics of male advertisement calls are important traits shaped by sexual selection and serve as signals for male quality and species recognition. Thus, anuran mating calls have been frequently used for studies of mate choice [e.g. 1-7] but also for taxonomic purposes and phylogenetics [e.g. 8, 9]. In several anuran taxa advertisement calls have helped in identifying cryptic species pairs [10, 11] and separating interspecific hybrids from their parental species [12]. Nevertheless, some authors have cautioned against the use of male calls for frog identification because of considerable within-taxon variation and great overlap in call features among hybrid and parental taxa [13, 14]. Major factors responsible for this call variation and overlap are differences in genotypes (1) breeding systems (2) and geographic and genetic distances (3).

1. Genotypes: An important genomic particularity that can alter phenotypic expression is polyploidization, which often comes along with hybridization and enables hybrids to overcome meiotic difficulties in order to successfully reproduce [15, 16]. Studies on call structure in polyploid anuran taxa of hybrid origin have revealed a causal relationship between ploidy and advertisement call structure [17-19]. Empirical studies using artificially created autotriploid and natural allotriploid Hylid frogs have shown direct effects of polyploidy on triploid male advertisement call structure [20] and even parallel developing call preferences in triploid females [21]. According to the results from these studies, changes in triploid male advertisement calls were causally related to a polyploidy-induced increase in cell size. In addition, phenotypic traits of polyploids can be expected to correlate with the relative numbers of the two parental genomes in the hybrid individual (“dosage effect”). Such correlations are well-known in plants [reviewed by 22]. In water frogs, [23] have recently demonstrated this for some morphological characters, but the combined results from other studies on water frogs yield no general support for the idea that traits of hybrid water frogs are shaped by dosage effects [reviewed by 24].
2. Breeding systems: Hybrids of different ploidies may further differ in call characteristics from their parental species and from each other as a result of various selection regimes, be it natural selection due to different acoustic environments or predator pressures [8, 25-27], be it sexual selection arising

from differences in mate choice preferences [28], or be it character displacement when different forms become reproductively isolated. Again, empirical support for such selection regimes is mixed. Some studies do find differences in advertisement calls and female preferences between polyploidy forms and their diploid relatives [29, 30], whereas others do not [11, 31, 32]. This is likely to reflect different selections pressures on male advertisement calls and female choice and, hence, can be expected to differ with the breeding system.

3. Geographic and genetic distances among populations: Interspecific hybridization can result in persistent call alterations in hybrids, due to genetic or chromosomal interactions that can cause changes in the morphology of the laryngeal apparatus [31], the nervous system [33], and the contractile frequency of muscles [34]. Given that hybridization is not uncommon in amphibians, it is likely to occur multiply across the area where two species overlap. When there is geographic variation in genetic, morphological, physiological and acoustical traits within the two parental species [as shown by 12, 35, 36], hybrids from different ancestral populations can be expected to produce different calls. On the other hand, genetic isolation by distance could cause populations of common origin to drift apart which will result in differences in several phenotypic traits, including advertisement call patterns [36, 37].

The study system

An excellent model organism for studying how these three factors influence advertisement calls is the Edible Frog *Pelophylax esculentus* (called *Rana esculenta* until [38]) *,* the most widespread and successful anuran hybrid in Europe. Its geographic distribution ranges from about 44° latitude in the south (southern France to northern Bulgaria) to 60° in the north (southwest Sweden to Baltic countries) and from the French Atlantic coast in the west to western Russia in the east (for details see Fig. 1.18 in [24]). The hybrid originally arose (and still arises) from interspecific matings between *P. lessonae* (Pool Frog) and *P. ridibundus* (Marsh Frog). The hybrid has abandoned the normal inheritance pattern of chromosomes and developed alternative ways of gamete production that circumvent incompatibilities between the parental genomes during meiosis. The typical and most widespread way is

hybridogenetic (= hemiclonal) reproduction, meaning that one of the parental genomes is excluded prior to meiosis and the other one clonally transmitted to haploid eggs and sperm, respectively [39, 40]. Hybridity and the diploid state are restored by back-crossing with the parental species whose genome was excluded. Depending on the specific genetic interactions between the hybrid and the parental species, three major breeding systems can be distinguished: the L-E-, R-E- and E-E-system [24, 41-43]. In the so-called L-E-breeding system (referring to the Latin names *lessonae* and *esculentus*), the excluded genome is that of *P. lessonae*, whereas in the R-E-breeding system (for *ridibundus-esculentus*), the *P. ridibundus* genome is excluded. In both cases, the hybrid has to live in sympatry and mate with one the respective parental species to regain the previously eliminated genome for its offspring. In these two breeding systems, all individuals are diploid and hybrids can only produce viable offspring when mating with the parental species, since crosses between two hybrids are usually lethal (Fig. 1a). Hence, the hybrid is a sexual parasite that needs a parental species as a sexual host for successful reproduction. At least for the L-E system this is also reflected in the mating behavior: both theoretical models and mate choice experiments have shown that diploid hybrid females (LR) should - and do - prefer LL males over their own [6, 44, 45].

In some populations this hybridogenetic mode of reproduction that is typical for diploid systems is modified in a way that hybrids have become entirely independent from the need to backcross with a parental species. As a result viable all-hybrid populations can exist. Such populations are concentrated in areas around the Baltic Sea, but also occur in some other areas of Europe [24, 46-52]. The explanation for the existence of such all-hybrid populations lies in the coexistence of diploid (LR) and triploid (LLR, LRR) animals in the same population [53]. The best-described diploid-triploid all-hybrid population system is the so-called E-E-breeding system (in reference to successful *esculentus-esculentus* pairings). In the typical and most widespread case, diploid hybrids (usually females) produce diploid gametes that result in viable triploid offspring when they fuse with haploid gametes (Fig. 1b). These haploid gametes can either be provided by diploids (usually males) through the hybridogenetic mechanism described above or by triploids of both sexes that exclude the single-copy genome (R in LLR and L in LRR) before they recombine the two remaining homospecific genome copies (LL and RR, respectively) during a normal meiosis [54, 55]. Thus, in these mixed ploidy populations triploid hybrids adopt the

role as sexual hosts for the diploid hybrids that the parental species have in diploid L-E and R-E-systems. In these all-hybrid systems, occasional fusion of two diploid gametes results in tetraploids, but these appear to be extremely rare in natural populations and have not yet been investigated in terms of their reproductive mode [54, 56]. Triploid forms, on the other hand, are widespread, and their reproductive patterns have been studied intensely for a number of decades [54, 56-64], including the mating behavior which, in contrast to the diploid L-E-system, seems to be random. For E-E-systems both theoretical models and empirical studies have shown that no preference should exist in hybrid females; and apparently it does not [65, 66]. Within the parental species' distribution ranges, diploid-triploid *P. esculentus* populations often co-exist and interbreed with parental genotypes, thus forming mixed populations [24].

With its various hybrid genotypes, different breeding systems and wide geographic distribution, *P. esculentus* provides all the variation that is required for testing in the same organism how genotypes, breeding system and geography influence variation in male advertisement calls. This is what we attempted in this study, starting with predictions from the following three not mutually exclusive hypotheses:

1. Genotype hypothesis: With L/R genome ratios differing among genotypes, dosage effects predict a directional increase (or decrease) in call parameter values in the order LL–LLR-LR-LRR-RR (i.e. 1.00-0.67-0.50-0.33-0.00).
2. Breeding system hypothesis: As hybrid females in diploid breeding systems must choose partners of a parental species for successful reproduction, whereas those in all-hybrid breeding systems with mixed ploidy should not have a preference, we expect different selections pressures on male advertisement calls. Hence, the selection hypothesis predicts that calls of the same hybrid genotypes will differ with the breeding system.
3. Geographic hypothesis: Given the wide distribution range of *P. esculentus* across Europe, the geographic hypothesis predicts that hybrids of the same genotype from far apart populations will differ in their advertisement calls. These differences could be due to their supposed origin from multiple primary hybridization events between *P. lessonae* and *P. ridibundus* from different populations and/or a common origin followed by drift [36, 37].

To test the predictions from these three hypotheses, we compared call parameter variation between hybrids of different genotypes (1), from different breeding systems (2), and from far geographically apart populations (3). Further, we examined advertisement call variation on a population level against genetic and geographic distance between populations. To our knowledge, this is the first study on bioacoustic differences that includes both hybridogenetic and sexual populations of the same anuran hybrid complex and is able to compare different genotypes and hybrids of different ploidies over an extensive geographic scale and in a population genetic context. Previous studies have provided extensive data on the genetic and inheritance patterns in populations with different ploidies, but empirical data on phenotypic manifestations in triploid versus diploid water frogs, or in recombining versus hybridogenetically reproducing hybrids are restricted to cell planimetry and body morphology [23, 67-71]. Where vocalization and other behaviors were investigated and found to vary within hybrid lineages [72-74], these studies were mostly restricted to mixed populations of diploid *P. esculentus* and one or both of its parental species. The same is true for studies using male advertisement calls for distinguishing between water frog species and populations [12a, 36b].

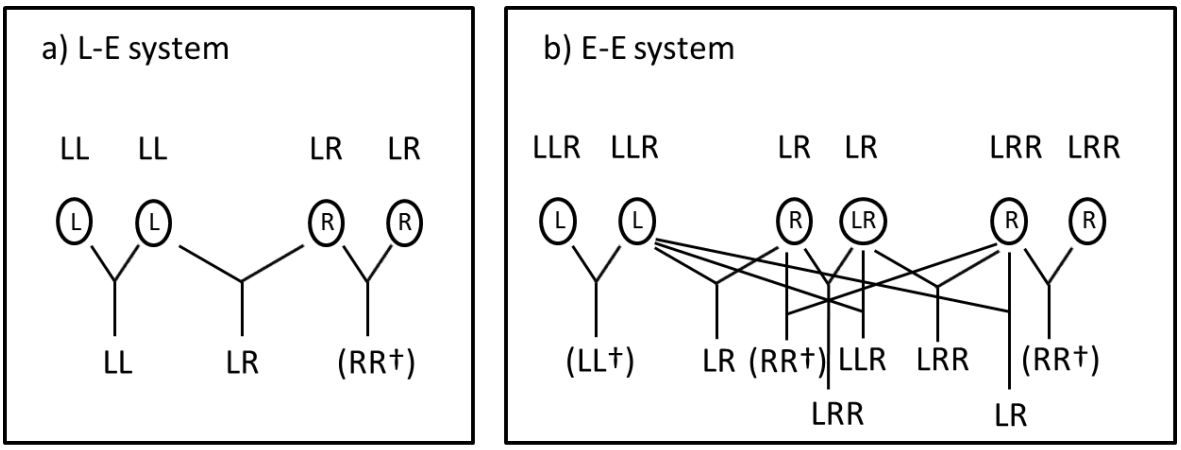


Figure 1. Overview of adult genotypes, gametes types (in circles) and resulting offspring. LL = *P. lessonae*, LR, LLR and LRR = *P. esculentus*. a) the L-E system, where only diploid hybrids are produced, and non-hybrid genotypes from matings between hybrids typically die prematurely. b) the E-E system, where all three hybrid types can cross, but only hybrid genotypes survive to reproductive maturity. In the L-E system, the R genome is never recombined, and L genomes are provided by *P. lessonae.* In the E-E system, three types of gametes are produced by hybrids and both L and R genomes regularly undergo recombination when they are present in double copy in triploid hybrids [54].

Methods

Selection of populations

In line with our three hypotheses, we recorded advertisement calls in nine populations with varying combinations of genotypes (hypothesis 1), breeding systems (hypothesis 2) and geographic distances (hypothesis 3). A map is shown in Figure 2. Choice of the study populations was based on relevant information from earlier studies [67, 75, Reyer unpublished data, 76, 77]. In terms of the breeding system, we differentiated between two hybrid systems, a) L-E and L-E-R populations, where diploid *P. esculentus* occur in sympatry with *P. lessonae* and/or *P. ridibundus*, produce haploid R gametes and backcross with *P. lessonae* (rarely *P. ridibundus*) and b) E-E and L-E-E populations, where some individuals form diploid gametes, and both diploid and polyploid hybrids either reproduce without backcrossing or by only occasionally mating with their parental species (usually *P. lessonae*). For ease of expression, we will refer to the populations described under a) as “diploid systems” (only haploid gametes and diploid hybrids are produced), and to the populations described under b) as “mixed ploidy systems” (due to the occurrence of diploid gametes, both diploid and polyploid hybrids can be produced).

In the three geographically distant diploid systems, diploid *P. esculentus* are sympatric with one parental species (*P. lessonae,* Hellberg) or both (*P. lessonae* and *P. ridibundus,* Herzberg and Šaštin-Stráže) (Table 2). Among the six populations of mixed ploidy, three contained diploid and triploid hybrids only, while in the other three (Altenhausen, Teschendorf and Kyritz) very few *P. lessonae* males and females were found (6 out of 147 individuals in total). In five of the six mixed ploidy populations all three hybrid genotypes (LR, LLR, LRR) were found at least in one sex; in the sixth population (Kozi chrbát, Western Slovakia) only LLR males and LR females were caught on this and one other occasion. Triploid LRR hybrids and LLR females are not known from this region, but LR males probably exist (Pruvost et al, subm.). In Teschendorf, only LR and LLR males have been found, although LL males are known to be present in this population (J. Plötner, pers. comm.).

Due to high variation in abundance, not all occurring types could be recorded within each population, and not in equally high numbers. In mixed-ploidy systems this was especially true for LRR males which were extremely rare in most populations, with the exception of Döbern (Table 2). In Altenhausen and Kyritz, single LL males have been observed but could not be recorded.

Figure 2. Map showing geographical locations of recorded populations. Dots = diploid populations with diploid hybrids and one or both parental species; triangles = mixed ploidy populations with mainly diploid and triploid hybrids, sometimes in sympatry with very few P. lessonae. For absolute numbers see Table 2.

Field work

In early summer of 2009, 2010 and 2011 we collected field recordings of male advertisement calls from the selected populations (Table 1). In Döbern and Genarp, we individually marked males using elastic and degradable waist-bands with clearly legible numbers to make them identifiable during repeated recordings. In other ponds we recorded calling males first and captured them directly afterwards for body size measurements and genetic identification. Additionally, some males were brought to Zurich and recorded in semi-natural outdoor ponds. During all recordings, focal males were recorded from a distance of 50-100 cm with microphones attached to a 1.5m bamboo stick and a hand-held digital recorder (Zoom H4n). We used a set of two mono-channel condensor microphones (AKG C417PP), one directed at the frog and the other attached to the observer. The two channels enabled separate recordings of

frog calls and observer comments on the caller's identity, allowing later distinction of simultaneous calls by several males in dense choruses. We recorded water temperatures just below surface level close to calling individuals. Most recordings were taken during peak calling activity, which usually takes place at water temperatures ranging from 17.5-22°C [78]. In our study, mean water temperature was 21.7 ± 2.0 °C (S.D., range 16.0-28.7 C).

Table 1. Population systems, number of recordings per genotype type and geographic coordinates of study populations. Recording numbers equal sample sizes per genotype for all analyses including call parameters.

Breeding system	Recordings per genotype					Population	Coordinates	
	LL	LLR	LR	LRR	RR			
Diploid	3		3			Hellberg (CH)	'47°17'45.72"N'	'8°48'48.38"E
	6		5		6	Herzberg (D)	51°37'36.66"N	'10°21'15.06"E
	1		4		2	Šastín-Štraze (SK)	'48°37'54.61"N	'17°8'40.38"E
Mixed Ploidy		6	8			Altenhausen (D)	52°16'40.00"N	11°15'15.00"E
		6	2			Teschendorf (D)	52°51'53.03"N	13° 8'40.38"E
		4	6	4		Kyritz (D)	52°54'07.08"N	12°19'15.50"E
		5	7	2		Genarp (SE)	55°36'34.00"N	13°23’19.00"E
		6	6	6		Döbern (D)	51°36'38.22"N	14°36'15.60"E
		6				Kozi chrbát (SK)	'48°37'53.58"N	17°17'41.28"E
Total (n = 104)	10	33	41	12	8			

Advertisement calls

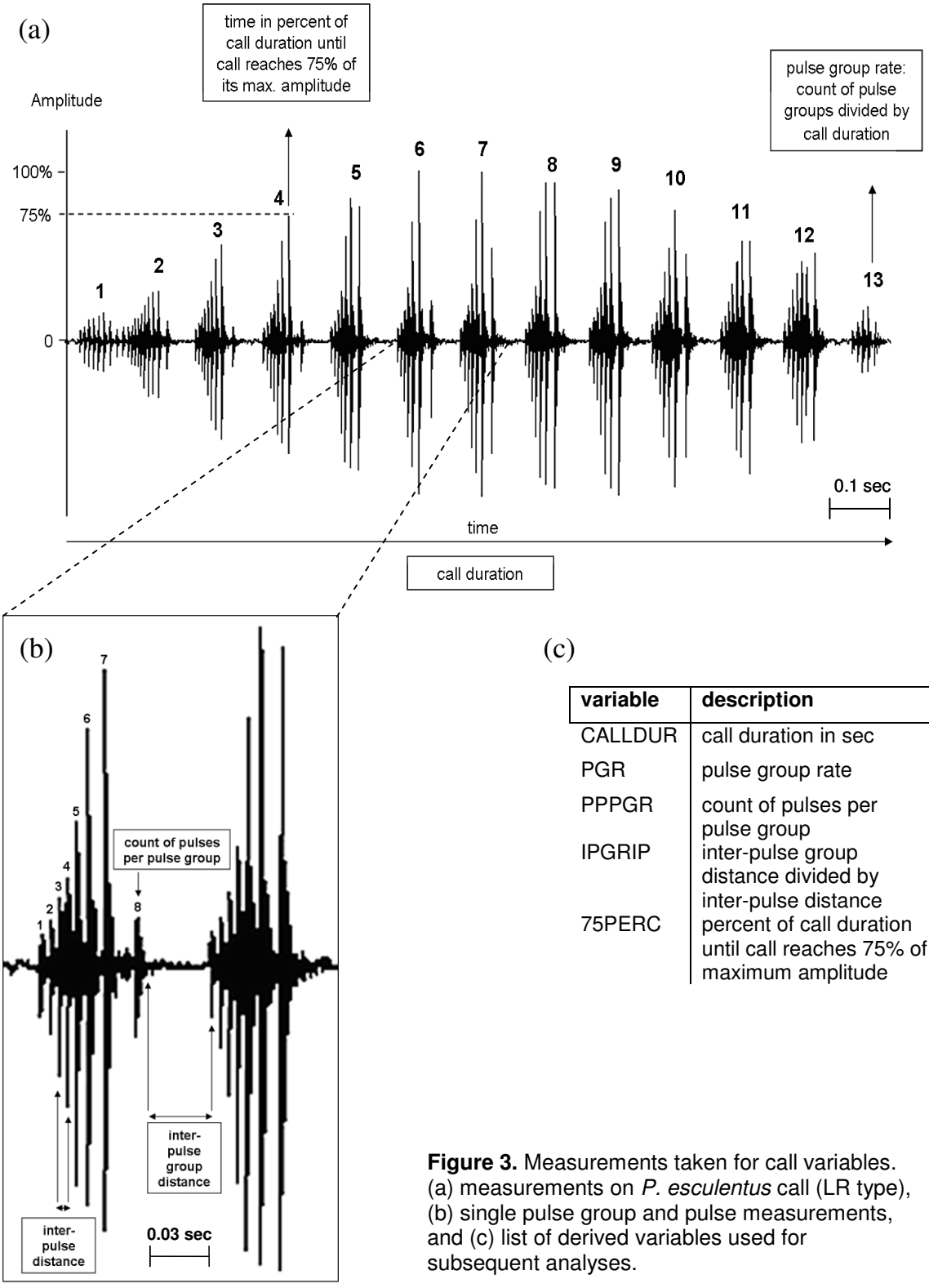
Water frog advertisement calls comprise a number of single pulses, which are bundled groups of varying distinction (Figure 3a). We defined the parameter pulse group (PGR) as a visible structure in the pulse sequence of a call. This structure can be either temporal (i.e. through long intervals between groups of condensed pulses) or energetic (i.e. through regular differences in amplitude that cause a visible pattern, although between-pulse group intervals can be short). For characterizing the temporal quality of the call, we used the following parameters: the entire length of the call (CALLDUR), the rate of pulse groups divided by the length of the call (PGR), the number of pulses per pulse group (PPPGR) and the ratio of inter-pulse group intervals to inter-pulse distance (IPGRIP). The latter describes the shape or “condensation” of pulse groups along the time axis. Energetic properties were expressed by the percentage of call duration that passes until the call energy rises from 10% to 75% of its maximum amplitude (75PERC). Although the rise from 10%

to 90% is a more conventional measure (C. Gerhardt, pers. comm.), we used the smaller range, because the two measurements are strongly correlated and the 10%-75% measurement showed fewer outliers and more normal data distribution. Variables CALLDUR, PGR and 75PERC yielded one value per call, while the pulse-group-based parameters PPPGR and IPGRIP were averaged over 4 measurements taken at regular intervals over the entire call. We generally measured and averaged 6 calls of good recording quality per individual. For 20% (21/104) of the males, averages could only be taken from 3-5 calls per individual.

Calls were cut and edited using the program ACOUSTICA 4.0. Call parameters were selected and measured in the program Avisoft SASPro. For comparative analyses between populations and genotypes we used the five above described temporal and energetic call parameters, which have been successfully applied to discriminate among anuran calls in other studies [20, 29]. We did not include spectral properties of the calls, since they can be strongly affected by calling context [79], which is difficult to quantify and, hence, was not recorded.

Population composition and genetics

Since the number of sound-recorded individuals per population was too low to calculate meaningful genotype ratios and population genetic parameters, we included additional samples from both males and females for most populations; these were collected at the same or a previous time for a different study with the aim of identifying genotype and sex ratios at these sites (see Table 2). Frogs were collected by hand or with a net, and a tissue sample (toe clip) was taken upon capture. To specify the genotype we used species- and genome dosage-specific allelic information from microsatellite markers. For this, DNA from ethanol-stored toe clips was extracted using the Qiagen BioSprint 96 DNA Blood Kit and the corresponding tissue extraction protocol. With each tissue sample two multiplex PCRs were conducted with 9 primer pairs each. Two loci were problematic due to amplification problems across all populations (one due to unambiguous allele specificity) and therefore the primers were excluded. Among the remaining 16 primers, 4 amplified the L, 8 the R and 4 both genomes (for details see Appendix 1). Those with markers for both genomes showed dosage-effects that were used to distinguish between LLR, LR and LRR by the relative density of the amplified species-specific alleles [80, 81]. In total, the 16 primers amplified 13 loci for each genome.



variable	description
CALLDUR	call duration in sec
PGR	pulse group rate
PPPGR	count of pulses per pulse group
IPGRIP	inter-pulse group distance divided by inter-pulse distance
75PERC	percent of call duration until call reaches 75% of maximum amplitude

Figure 3. Measurements taken for call variables. (a) measurements on *P. esculentus* call (LR type), (b) single pulse group and pulse measurements, and (c) list of derived variables used for subsequent analyses.

Alleles were scored in the program Genemapper (Applied Biosystems 2004, Genemapper vers. 3.7.). Most alleles could be assigned unambiguously to either the L or the R genome, and individuals showed a clear “consensus genotype”, i.e. the same genotype for each of the used microsatellite markers. We checked the data set for existing null alleles separately for the L and R genome. Null alleles are unmasked and can be easily detected in the hemizygous state of LR hybrids and in single genome copies of triploids (R in LLR, L in LRR) (for details see [55]). Correspondingly, null alleles can potentially be masked in homozygous individuals of the parental species and triploid hybrids carrying two copies of the genome in question. Populations and loci where no unmasked null alleles were detected we considered null-allele-free. In few cases of null alleles (< 2 cases per population), the individual was excluded from the data set. At two different loci (RlCA1b6 and Re1Caga10), more than 2 cases of unmasked null alleles were found in one and two populations, respectively. This means that a relevant number of undetected null alleles might exist in these populations and could potentially bias population genetic estimates. At locus RlCA1b6, all individuals of the concerned population (Altenhausen) were hemizygous (either LR or LLR with unmasked null in the R genome). Thus, the null allele could always be detected and was therefore coded as a real allele. However, at locus Re1Caga10 both hemi- and homozygous individuals of two populations (Šaštin-Stráže and Teschendorf) were affected (LL and LR, null occurring in the L genome). Therefore, the entire locus was recoded as missing data and not used for analysis.

Genetic variation was expressed by gene diversity (H_e), which is gene variability corrected for sample size, and Nei’s D [82], both calculated in the program SPAGeDi version 1.3. SPAGeDi accepts haploid and diploid individuals in the same analysis under the assumption that the two genomes in diploid individuals recombine. The program also requires individuals to have only one genome type, which is violated in hybrids. To circumvent this problem, we separated the L and R genome data into two different input files and analyzed them with a method, that has been successfully employed by other authors [46, 52, 54, 58, 75]: LR hybrids were treated as haploid for both genomes, LLR and LRR hybrids were treated as haploid for the single haploid and diploid for the double genome, and the parental species (LL and RR) were treated as diploid for the L and R genome, respectively.

Table 2. Genotype distribution, number of genetic samples and genome-specific gene diversity He in diploid an and mixed ploidy breeding systems. Gene diversity He is based on allele frequencies in L and R genomes, corrected for sample size (Nei 1978).

Population	Ploidy types	Counts per type		N genetic samples (all types)		He (all types)	
		♂	♀	L	R	L	R
Diploid systems							
Hellberg	LL	11	14	14	3	0.67	0.06
	LR	3	6				
Herzberg	LL	9	2	21	22	0.45	0.40
	LR RR	12	3				
		10	-				
Šastín-Štraze	LL	4	1	48	49	0.5	0.45
	LR RR	27	16				
		2	4				
Mixed ploidy systems							
Altenhausen	LL LLR	3	1	29	26	0.36	0.53
	LR	17	5				
	LRR	8	1				
		1	-				
Teschendorf	LL LLR	-	1	34	33	0.37	0.35
	LR	21	2				
	LRR	2	4				
		-	1				
Kyritz	LL	1	-	81	80	0.34	0.40
	LLR	9	4				
	LR	18	20				
	LRR	10	19				
Genarp	LLR	14	4	175	175	0.15	0.12
	LR	106	24				
	LRR	3	24				
Döbern	LLR	33	3	149	149	0.16	0.30
	LR	24	23				
	LRR	48	17				
Kozi chrbát	LLR	89	-	105	105	0.29	0.39
	LR	-	16				

Statistical analyses

For testing whether and how the five call parameters mentioned above differ among genotypes and localities we performed a separate GLM for each parameter. Since several call properties are influenced by body size and water temperature [31, 83, 84], we included these variables as covariates. All GLMs were perfomed in a stepwise manner with backward elimination; starting with the full set of predictive variables, we successively dropped those with a probability > 0.05. Effect size, i.e. the strength of the association between a significant predictive variable and the dependent variable, was calculated as $\eta^2 = SS_{effect}/SS_{total}$, where η^2 is the proportion of the effect variance (SS_{effect}) to the total variance (SS_{total}). Conventional critical values for small, medium and large effect sizes are 0.10, 0.25 and 0.40, respectively [85].

For investigating how genotypes differed in their overall mating call structure, we performed a discriminant analysis on the set of all five call parameters with genotype as the separating factor. Based on the results from this analysis, we averaged the first two canonical scores for each genotype from each population. From these averages we created a matrix of pairwise Euclidean distances as an index of average call dissimilarity (also referred to as call distance in the following). This measure was used to structure the different genotypes of all populations in a hierarchical cluster analysis implementing the group average linkage type method [86]. For a subsequent two-sample comparison between two different breeding systems, we used a multivariate Hotelling's T-test with 10000 random permutations, since the low number of sample sizes was inadequate for a discriminant analysis. To perform multiple correlation analyses we created different pairwise distance matrices.

To address the question whether call variation patterns were correlated with geographic distance and/or genetic distance (a link to be expected under isolation by distance), we performed Mantel tests, based on genotype-specific subsets of call distance and genetic distance data. For a matrix of geographic distances, Euclidean distances between sampling sites were calculated from GPS coordinates into inter-population distance data (km) using an online GPS Latitude and Longitude Distance Calculator (www.csgnetwork.com/gpsdistcalc.html). For genetic comparisons, pairwise matrices of Nei's D were created for the L and R genome separately, based on the full sample size (including all genotypes that carry the genome in question) for each population, using the program SPAGeDi. Pairwise distance matrices of

geography, call dissimilarity and Nei's D values were then correlated with simple and partial Mantel tests in the program zt 1.2 [87] with 1000 permutations. Simple Mantel tests were performed to find correlational relationships between two pairwise distance matrices, and partial Mantel tests were used to control for potential covariation by a third distance variable matrix [88]. Earlier studies on polyploid water frog populations had indicated that such covariance might occur between genetic distance and geographic distance and potentially influence call distance. Significance tests were computed by running 1000 iterations of the data set. To avoid mixing populations of different reproductive modes, we restricted these analyses to a subset of populations containing both LR and LLR individuals. Unfortunately, the number of populations containing only diploid hybrids and those containing LRR hybrids were too low to perform Mantel tests for these groups.

Unless otherwise stated, statistics were performed in the programs NCSS [89] and SYSTAT 11 [90].

Results

Differences in single call parameters

Variation in each of the five call parameters was investigated in relation to two categories (genotype and population) and two covariates (water temperature during calling and male body size) by means of GLMs. Results are shown in Table 3 and Fig. 4. Among the covariates, body size had no effect on any of the five call parameters and temperature influenced only three of them: CALLDUR decreased and PGR and IPGRIP increased with increasing temperatures. However, although significant, the size of the temperature effect was low for all three parameters explaining only 12%, 7% and 1% of the variation, respectively (see η^2 values in Table 3). The strongest and most consistent effect on call parameters was exerted by genotype, which showed a significant influence on all five call parameters. Differences among genotypes were always directional, with means either increasing (PPPGR, IPGRIP) or decreasing (CALLDUR, PGR, 75PERC) in the order LL, LLR, LR. LRR, RR, i.e. with the ratio of L/R genomes (1.00, 0.67, 0.50, 0.33, 0.00). LL calls were of longer duration, higher pulse group rate and reached 75% of the maximum amplitude later than RR calls, whereas the number of pulses per group and the ratio of inter-pulse group intervals to inter-pulse distance were higher for RR than for LL. For CALLDUR and 75PERC, there was an almost linear decrease from LL through hybrids to RR, whereas for PGR hybrid means were slightly closer to RR and for IPGRIP and PPPGR closer to LL. Bonferroni posthoc tests revealed the following significant pairwise differences: all five genotypes differed from each other in IPGRIP and all but LLR and LR also in PGR and PPPGR. For the remaining two parameters only the most extreme pairs differed significantly: LL from LRR and RR in CALLDUR and LL – by trend also LLR (P=0.075) - from RR in V75PERC. Together with the fact that IPGRIP also had the highest effect size (0.86), these results indicate that this variable differentiated best between genotypes.

For two call parameters (PPPGR and IPGRIP) there were also significant population effects (Table 3). Posthoc pairwise comparisons showed that it was mainly the northernmost population of Genarp that differed from the rest. Here, mean values for both call parameters were lower than in the other populations. Moreover, a separate analysis for this population revealed no significant differences between the three hybrid types (LLR, LR, LRR) for any of the five call parameters (all $F_{2,9} \leq 0.693$, all $P \geq 0.525$).

Table 3. Results from GLMs testing for the effects of two categories (genotype and population) and two covariates (water temperature and male body size) on five call parameters. Shown are F-ratios, P values and effect sizes (η2) for significant relationships.

Call parameter	Statistics	Genotype	Population	Temperature	Body size
CALLDUR	F	4.348		15.398	
	P	**0.003**		**<0.001**	
	η2	0.132		0.117	
PGR	F	84.221		35.314	
	P	**<0.001**		**<0.001**	
	η2	0.715		0.075	
PPPGR	F	54.368	6.532		
	P	**<0.001**	**<0.001**		
	η2	0.601	0.144		
IPGRIP	F	207.650	4.616	5.305	
	P	**<0.001**	**<0.001**	**0.024**	
	η2	0.862	0.038	0.006	
75PERC	F	7.697			
	P	**<0.001**			
	η2	0.237			

Overall call differences

For analyzing the overall call differences among the two parental and three hybrid taxa, we subjected all five call parameters to a discriminant analysis with genotype as the separating category. We obtained four discriminant functions, but only the first two functions were significant (Table 4 a). Together they accounted for more than 99% of the total dispersion. Function 1 had the strongest influence on data separation (83.4% of total dispersion). Correlations of function 1 with the five call parameters mirrored the results from preceding univariate analyses. Function 1 was positively correlated with IPGRIP (r = 0.77) and PPPGR (r = 0.37), and negatively correlated with PGR (r = -0.43), CALLDUR (r = -0.13) and 75PERC (r = - 0.13). Correlations between parameters and discriminant function 2, which accounted for 16% of total dispersion, were positive for all call parameters (IPGRIP: r = 0.43; PPPGR: r = 0.21; CALLDUR: r = 0.18; 75PERC: r = 0.12), and strongest for PGR (r = 0.55). Again, IPGRIP was the call variable with the highest power of discrimination for genotype as a grouping factor. A scatter plot of the first two canonical functions is shown in Figure 5, illustrating that the discriminant analysis results based on our five parameters resulted in little overlap between genotypes.

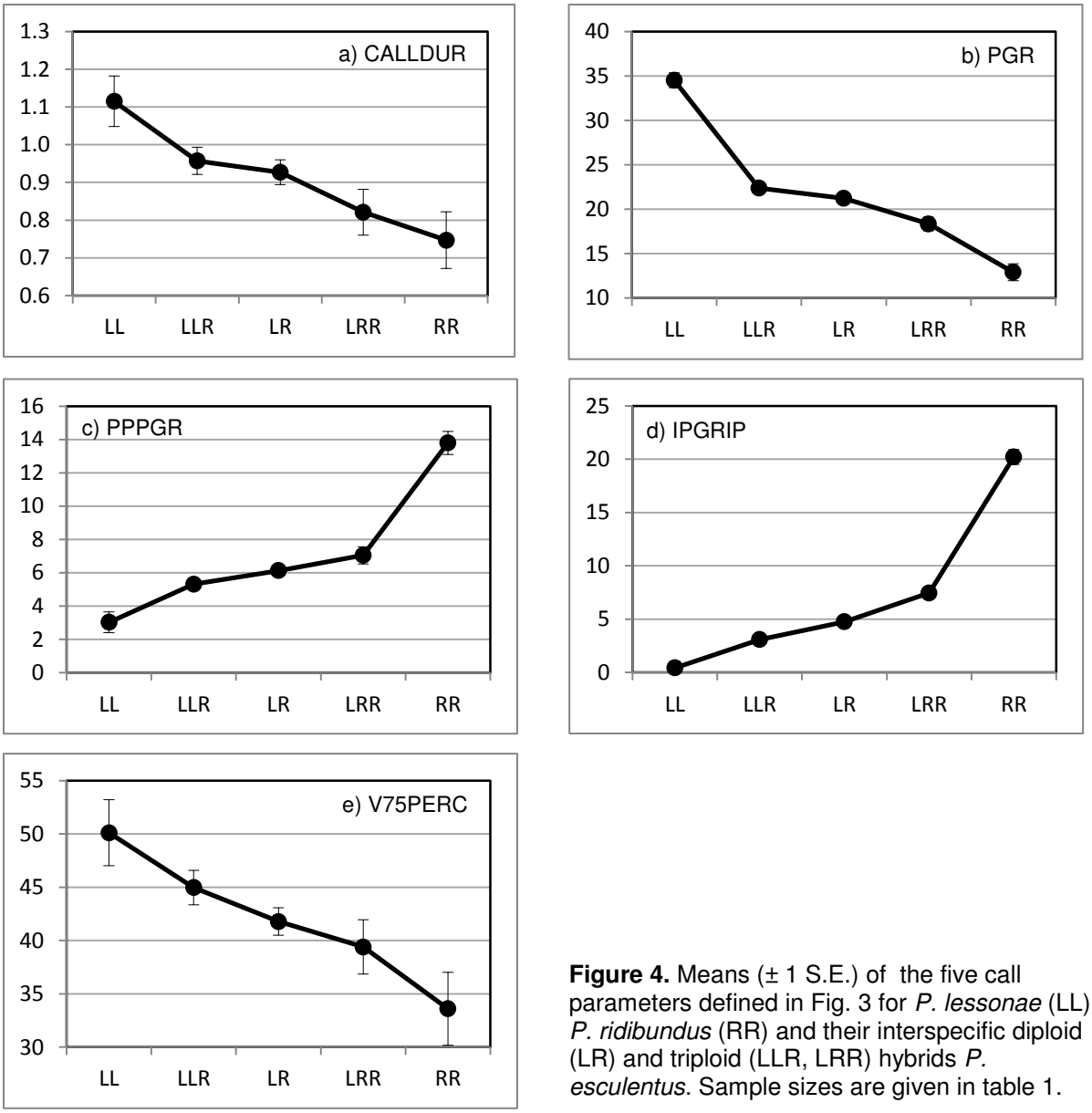


Figure 4. Means (± 1 S.E.) of the five call parameters defined in Fig. 3 for *P. lessonae* (LL), *P. ridibundus* (RR) and their interspecific diploid (LR) and triploid (LLR, LRR) hybrids *P. esculentus*. Sample sizes are given in table 1.

Classification of individuals was highly successful, with an overall 88.5% (92 out of 104 individuals) classified to the correct genotype (Table 4 b). One hundred percent correct classification was achieved for the two parental types RR and LL, whereas correct classification of hybrids was only between 80 and 90%. LLR hybrids were three times misclassified as LR and once as LRR, and LRR were twice misclassified as LR. Diploid LR hybrids were six times misclassified as triploids, three times each as LLR and LRR.

Table 4. Results of multivariate discriminant analysis with genotype as discriminating factor.

(a) Canonical variate analysis for discriminate functions. Significant functions are printed in bold.

Function	Eigenvalue	% of total dispersion	Canonical correlation	Wilk's lambda	F	df	P
1	**15.52**	**83.4**	**0.969**	**0.013**	**41.9**	**20**	**< 0.0001**
2	**2.98**	**16.0**	**0.865**	**0.225**	**16.0**	**12**	**< 0.0001**
3	0.09	0.5	0.299	0.898	1.8	6	0.11
4	0.01	0.1	0.114	0.986	0.6	2	0.52

(b) Classification matrix

	Actual	Predicted				% Correct
	LL	LLR	LR	LRR	RR	
LL	10	0	0	0	0	100.0
LLR	0	29	3	1	0	87.9
LR	0	3	35	3	0	85.4
LRR	0	0	2	10	0	83.3
RR	0	0	0	0	8	100.0

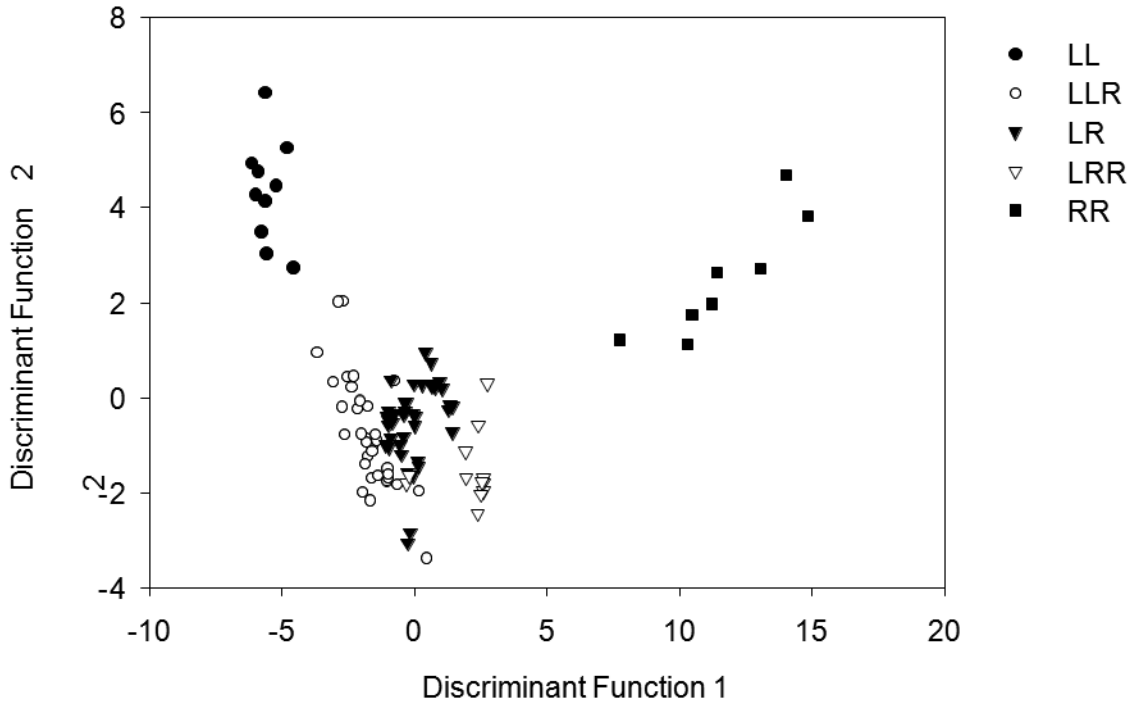


Figure 5. Discriminant functions plot of water frog calls. Canonical scores 1 and 2 were calculated from the combined parameters CALLDUR, PGR, PPPGR, IPGRIP and 75PERC.

Call structure in relation to breeding systems

Although with 85% classification success for diploid hybrids was fairly high, it was not perfect. We therefor tested whether some of the unexplained variation might be caused by the affiliation of diploid hybrids with one or the other of the two earlier defined breeding systems, i.e. whether LR calls differ between diploid populations, where successful hybrid reproduction requires preference for and mating with individuals of a parental species, and mixed ploidy systems which can be maintained by random
hybrid x hybrid matings. Since the overall sample size of LR hybrid calls was too low to perform a reliable discriminant analysis between the two breeding systems, we performed a multivariate Hotelling's T-test with 10000 random permutations instead. The test results show a highly significant difference between diploid and mixed ploidy systems when combining all call characteristics for LR hybrids (All variables combined: Hotelling's $T^2_{5,31} = 189.05$, P = 0.0001). In subsequent parametric T-tests using all five variables individually, only two variables turned out discriminative: PPPGR ($T_{2,31} = 3.39$, P = 0.002) and IPGRIP ($T_{2,31} = 8.31$, P < 0.0001). For both variables, mean values of LR calls from diploid systems were less similar to *P. lessonae* than those from mixed ploidy systems. Although not significant, there was an indication that this shift towards the *P. ridibundus* pattern was mainly in the populations of Herzberg and Šaštin-Stráže, where both parental species occur, whereas in Hellberg where *P. ridibundus* is absent values for LR were not different from the overall averages shown in Figs. 4c and d (Herzberg: PPPGR = 8.86 $\pm$ 1.16, IPGRIP = 10.47 $\pm$ 2.04; Šaštin-Stráže: PPPGR = 7.71 $\pm$ 1.80, IPGRIP = 9.17 $\pm$ 3.14; Hellberg: PPPGR = 5.72 $\pm$ 1.94, IPGRIP = 4.02 $\pm$ 3.43; means $\pm$ SE). The means of the remaining variables did not differ between the two breeding systems (CALLDUR: $T_{2,31} = 1.59$, P = 0.12; PGR: $T_{2,31} = 0.67$, P = 0.51; 75PERC: $T_{2,31} = 0.96$, P = 0.34).

Call structure in relation geographic and genetic distances between populations

A hierarchical cluster analysis based on Euclidian distances between the two significant discriminant functions was performed to examine and visualize call similarities, respectively distances, in relation to population, i.e. geographic location. In the resulting dendrogram (Fig. 6), hybrid *P. esculentus*, parental *P. ridibundus* (RR) and *P. lessonae* (LL) formed three main clusters. RR populations showed higher dissimilarity among themselves than LL populations, which were positioned

closer to the hybrid than to the RR cluster. Within the hybrid cluster, LR, LLR and LRR formed separate and largely homogenous groups. These were independent from the population of origin, thus showing clear genotype-specific separation. Hence, call similarities are higher between same-genotype groups from different populations than between different genotypes from the same population. The only exception from this pattern was found in calls from the northernmost mixed ploidy population Genarp (southern Sweden). Here, calls of diploid and triploid frog types were nested in the larger LR cluster. Thus, LLR and LRR calls were more similar to each other and to sympatric LR calls than to calls of the corresponding triploids from other populations. This is consistent with the above described results from the univariate analyses of differences in single call parameters.

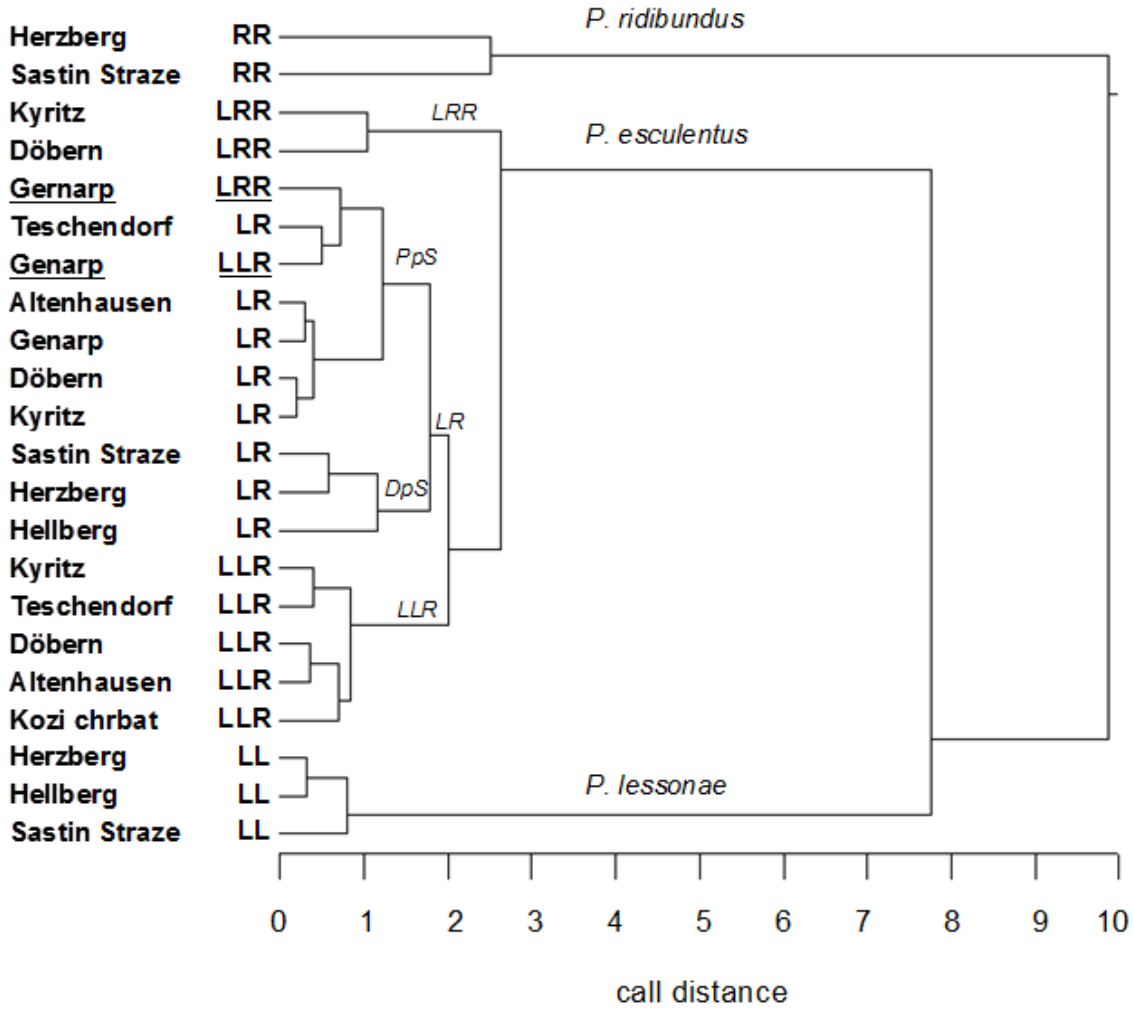


Figure 6. Cluster dendrogram calculated from Euclidean call distances (based on discriminant analysis scores) between genotypes from different populations. Given in italics are species names, P. esculentus ploidy types (LRR, LR, LLR), and population systems within the LR group (Pps = polyploid system, DpS = diploid system). Two groups (LLR and LRR from the most northern population of Genarp) are underlined to indicate their exceptional behavior within the overall pattern of genotype-specific clustering.

For a direct comparison of genetic diversity between diploid and mixed ploidy systems we used gene diversity corrected for sample size (Nei 1978) for the L genome (He_L) and R genome (He_R), respectively. Values of He for each marker were averaged for each genome. Mean gene diversity in the L genome (He_L) was higher in diploid systems than in mixed ploidy ones ($T_{2,9}$ = 3.21, P = 0.01), but the two systems did not differ in gene diversity in the R genome (He_R; $T_{2,9}$ = -0.30, P = 0.77). However, it should be considered that the value for He_R of one diploid population (Hellberg) was calculated from a very low sample size of individuals carrying an R genome (Table 2). Therefore, results for this population should be interpreted with caution.

Under the assumption that phenotypic data like differences in call characteristics should not influence isolation-by-distance in either genome, we performed simple Mantel tests between geographic distance and genetic distance for both genomes without controlling for a third matrix. These tests yielded no significant correlation between geographic distance and genetic distance (*R genome*: r = 0.33, P = 0.22; *L genome*: r = 0.02, P = 0.48). Subsequently, we tested whether call distances between populations within the same genotype were correlated to genetic and geographic distance by performing partial Mantel tests. These tests included call distance as a third matrix and alternatingly controlled for genetic and geographic distance, since these variables might still interact in their influence on call differences. In both LR and LLR hybrids, call similarity and genetic distance in the R genome did not correlate significantly after controlling for covariance by geographic distance (*R genome:* LR: partial r = 0.50, P = 0.2, LLR: partial r = -0.57, P = 0.14; *L genome:* LR: partial r = 0.10, P = 0.41). In the reverse tests, when controlling for genetic distance, we found no significant correlations among LR hybrids (*R genome:* LR: partial r = 0.08, P = 0.5; *L genome:* partial r = 0.09, P = 0.47). Among LLR hybrids, we did find some – yet non-significant – indication for a correlation between call distance and geographic distance when controlling for the R genome (partial r = 0.75, P = 0.10) and for the L genome (partial r = 0.63, P = 0.13).

Thus, there was no indication for a pattern of genetic isolation by geographic distance in the L and R genome, nor did call differences correlate with geographic or genetic distance.

Discussion

Genotype differences in male advertisement calls

In our study on male advertisement calls from nine *P. esculentus* populations across a broad geographic scale, we found significant genotype effects on all five call parameters that we considered. The strength of the genotype effects, however, varied among the call parameters. Effect sizes were small to medium for call duration (CALLDUR) and duration until 75% of the maximum amplitude was reached (V75PERC); large effect sizes were found in pulse group rate (PGR), the number of pulses per pulse group (PPPGR) and the condensation of pulse groups within the call (IPGRIP). These results from the univariate analyses of single call parameters were fully supported when all call parameters were combined in a multivariate analysis (Fig. 5). Again, genotype discriminated very well between call properties, with 88.5% of all individuals correctly assigned. Triploid hybrids (LLR and LRR) scored closer to diploid hybrids (LR) than to their double-genome parental species, and were wrongly assigned only to other hybrids, but never to parental genotypes. A cluster analysis confirmed that, overall, call similarity was a strong population-independent separator among genotypes (with one exception, see below). The best separating parameter was IPGRIP. In the univariate analysis it had the largest effect size Table 3) and in the multivariate analysis the highest correlation ($r = 0.77$) with discriminant function 1. This function did not only clearly differentiate between the two parental species but also fairly well between the three hybrid types (Fig. 5).

Earlier studies, partly using different call properties than we did, have already shown a robust differentiation between diploid *P. esculentus* and its parental species [12]. Our findings support this for additional call parameters, but – more importantly – they also reveal a differentiation between syntopic triploid and diploid hybrids for most mixed ploidy populations we sampled. For all five call parameters (Figs. 4a-e) and for the combined data set (Fig. 5) values either decreased or increased in the order LL-LLR-LR-LRR-RR, i.e. with L/R genome ratios of 1.00-0.67-0.50-0.33-0.00. This is in full agreement with expectations from a genomic dosage effect.

So far, evidence for a dosage effect on water frog traits is mixed [reviewed by 24]. In a recent study comparing morphological differences between LLR, LR and LRR hybrids and their LL and RR parental species, [23] found that differentiation in morphological indices are directional in the order LL-LLR-LR-LRR-RR, but the influence of the L haplotype was greater than the influence of the R haplotype. Thus,

all hybrid types (including LRR) were morphologically closer to *P. lessonae* than to *P. ridibundus*. Conversely, [91] found that in triploid LLR hybrids most (but not all) morphological, ecological and biochemical traits resemble *P. ridibundus* more than *P. lessonae*, although the hybrids possess two LL and only one R genome. He explained the deviation from the expected dosage effect by "genomic imprinting", i.e. the overexpression of R and/or repression of L genes in offspring through the maternally inherited R genome. Although being more *P. ridibundus* like would be adaptive for LLR hybrids because it could help them in competition with RR males over access to RR females, the proposed imprinting mechanism cannot work. As [95] pointed out, natural selection cannot act on the LLR hybrids' R genome, because it is excluded from the germline. Similarly, LRR in our populations cannot become more *P. lessonae* like through natural selection on the L genome, because in this hybrid type, the L is excluded and, hence, an evolutionary dead end. Whatever the true genetic mechanism behind the deviation from dosage ratios (see [95] for alternative explanations), it cannot be denied that water frogs exhibit mosaic-like phenotypes with some traits shaped by genetic information in the double-copy part of the genome (LL) and others by the dominance of the single copy (R).

The results on call differentiation from our study are more in line with those of [23] on morphological characters: the double-genome appears to "pull" the phenotypic expression of the triploid hybrid in the direction of the respective parental species, as expected under the dosage effect hypothesis. This is obvious from the fact call parameter values are either decreasing or increasing in the order LLR-LR-LRR. For two parameters (PPPGR, IPGRIP), however, the dosage effect is skewed in direction of the L-genome, both in the univariate analyses (Fig. 4) and the discriminant analysis where along function 1 (mainly representing (IPGRIP and PPPGR) hybrids were located closer to *P. lessonae* than to *P. ridibundus* (Fig. 5). This suggests that even in the haploid state the influence by L is stronger than by R. Results from previous studies on *P. esculentus* and another hybridogenetic hybrid, *P. grafi* (a hybrid between the Iberian water frog *P. perezi* and *P. ridibundus* that hemiclonally transmits one copy of *ridibundus* genome), confirm that manifestation of call characteristics in both hybrid lineages converge towards the non-*ridibundus* genome [14]. However, for PGR the opposite was true: all hybrid types resembled *P. ridibundus* more than *P. lessonae*. This variation in the relative "strength" of L and R genomes suggest the existence of additional influences on calls, including the ones

discussed below: the breeding system and factors related to geographic distances between populations.

The role of the breeding system for advertisement call differences

While LR hybrids are considered to be phenotypically intermediate between the parental species LL and RR [12, 14] and between their triploid conspecifics LLR and LRR [23], we found considerable variation in the expression of call parameters among LR hybrids from different breeding systems. Compared to mixed ploidy populations, LR hybrids from diploid systems showed higher genetic diversity in the L genome and were less similar to *P. lessonae* in two highly discriminative call parameters (PPPGR and IPGRIP). This difference could possibly be explained by the particularities of genome inheritance in the two population types. LR hybrids from mixed-ploidy populations receive and pass on previously recombined copies of one or both genomes that descended from one diploid and one triploid, or from two triploid parents. LR hybrids from diploid systems, on the other hand, receive the premeiotically excluded genome from a parental species (*P. lessonae* in L-E-systems, *P. ridibundus* in R-E-systems); but they do not transfer it to the next hybrid generation. Because of this “dead end” there is – contrary to mixed ploidy systems - no selection on the “rented” parental L or R genome within the hybrid; selection in diploid systems occurs only in the parental species for which the hybrid’s “interest” is not relevant. The hybrid’s clonally transmitted genome, however, can be the subject of selection processes, if certain hemiclones are more successful than others as suggested by the frozen niche hypothesis [92]. This difference in selective processes suggests that in LR frogs, that exclude the L genome, the R genome may exert a slightly stronger effect on call parameters, with the result that PPPGR and IPGRIP of LR hybrids are slightly higher (and thus more *P. ridibundus* like) than in mixed ploidy systems, although overall they are still more similar to the *P. lessonae* pattern (Figs. 4a-e).

Given that previous studies have shown that hybrid and parental females in diploid hybrid systems prefer parental over hybrid males [6, 44, 45], it would be beneficial for LR hybrid males in diploid systems to sound like the parental species they co-exist and breed with. This seems to be supported by our results from diploid systems. In all three of them, LR hybrids exclude the L and clonally transmit the R genome [55, 93]. However, in two of them (Šaštin-Stráže and Herzberg), *P.*

esculentus co-occurs with both *P. lessonae* and *P. ridibundus*. Here, the comparatively greater similarity of LR calls to *P. ridibundus* (when compared to those from mixed ploidy systems) may be an adaptation of hybrid males to mimic *P. ridibundus* calls for a reproductive benefit when attempting to mate with *P. ridibundus* females. In fact, for these two populations, low pairwise F_{ST} values between LR and both LL and RR indicate that diploid hybrids are mating with both parental species [55]. In the L-E-system of Hellberg, however, where *P. ridibundus* does not occur and, hence, LR hybrids should mimic *P. lessonae* as much as possible, the shift towards *P. ridibundus* features does not seem to exist. Thus, in all three diploid populations, the basic mechanism is the same, namely selection between different clonal R lineages, but the outcome differs in agreement with the breeding system: it makes the hybrid calls similar to the calls of the parental species that can act as sexual hosts.

The role of geographic and genetic distance for advertisement call differences

In addition to the marked genotype effect, we also found a population effect on calls, although much smaller and for only two of the five parameters, PPPGR and IPGRIP (Table 3). These two parameters are the same that differentiate best between genotypes and are also influenced by the breeding system. This population effect is not surprising. According to previous studies, *P. esculentus* populations originated from multiple primary hybridization events in sympatric areas of *P. lessonae* and *P. ridibundus* with subsequent dispersal of different hybrid lineages [94, 95]. These lineages differ in several ways, including the abilities of the L- and R-genomes to induce and resist genome exclusion [95-97], the gamete production patterns and the way how triploids are formed (Pruvost et al., subm.). In light of these genetic differences and the large intraspecific variation in calls of the parental species *P. ridibundus* and *P. lessonae* across Europe [12, 35], it seemed plausible to hypothesize that some of the variation among hybrids from different localities has resulted from different call characteristics of the parental haplotypes that were involved in primary hybridizations. This is why we tested for possible spatial and genetic correlations with inter-population call dissimilarities. To avoid mixing populations with different breeding systems and because the number of populations containing only diploid hybrids and those containing LRR hybrids was too low, we restricted the corresponding Mantel tests to a subset of populations containing both

LR and LLR individuals. The tests neither revealed a genetic isolation by distance pattern, nor did call differences correlate with geographic distance.

There was one population in our study that deviated from the general pattern found in the other eight populations. In the northernmost all-hybrid population from Genarp (Southern Sweden), triploid LLR and LRR calls were similar to each other and lay embedded in the same cluster as LR from their own and from other populations (Fig. 5). This stood in sharp contrast to the other mixed ploidy populations, e.g. the pond in Döbern (East Germany), where all three hybrid types appeared in different clusters. The difference is also immediately obvious in representative call oscillograms from these two populations: they show clear differences between LLR, LR and LRR for Döbern, but similar patterns for Genarp (Fig. 7).

A proximate explanation for the lack of differentiation among calls by frogs from Sweden could be their comparatively low genetic diversity, which has been attributed to their location outside the distribution range of both parental species and close to the northern edge of the Central European distribution range of *P. esculentus* [56, 75]. Our results confirm this pattern: among the nine studied populations, *P. esculentus* from Northern Europe had the lowest genetic diversity (He) in the L genome and the second lowest in the R genome. If call differentiation has a genetic basis, a lack thereof among northern European frogs could have several possible explanations. For example, prior to the post-glacial colonization of the north one of the numerous primary hybridizations in Central Europe may have resulted in hybrids that lacked the call differences from the very beginning. Alternatively, a mutation may have disabled the expression of call differences either before or after the colonization. In the absence of parental genotypes, this novel genetic information may have been “frozen” [frozen niche variation model, see 92] in hybrid lineages that dispersed north. Finally, introgression of nuclear genes from *P. lessonae* into the *P. ridibundus* genome could also have caused a diminution of *ridibundus*-like call characteristics. Nuclear introgression has been found in a number of *P. esculentus* populations [98-101]. Regardless of the exact proximate mechanism, a lack of dosage-specific call differentiation would have gotten established in northern all-hybrid populations if it either turned out neutral (i.e. through genetic drift) or beneficial (e.g. through sexual selection).

An ultimate explanation for the lack of call differentiation in Swedish frogs may lie in the role of male vocalization in female mate choice. From an evolutionary perspective, discrimination of male calls makes sense in diploid populations where hybrid females suffer a severe reproductive disadvantage from mating with hybrid males, since their common offspring are usually unviable due to the accumulation of deleterious alleles in the hybridogenetically transmitted R genome [102, 103]. In contrast, genetic fixation of mate preferences in a particular genotype should be impossible in diploid-triploid all-hybrid populations where suitable partners alternate each generation: diploid LR females producing diploid eggs should choose triploid LLR or LRR males; the resulting triploid daughters should choose diploid LR males etc. [66]. Results from playback experiments are consistent with these predictions. *P. esculentus* females from diploid populations prefer calls of *P. lessonae* over those of their own hybrid males [6, 44, 45]. In contrast, female *P. esculentus* from Sweden did not show any ploidy-specific preference of male advertisement calls [65]. Whether this lack of discrimination reflects that they should not (ultimate reason), or that they cannot differentiate between males of different ploidy, because their calls do not differ much (proximate reason), remains an open question. At present, we also do not know why considerable ploidy-specific call differences do exist in other mixed ploidy hybrid populations (e.g. Döbern), where – from an evolutionary point of view - they also should not play a role in mate choice. Whether in these populations females do use the existing call differences to choose between males of different ploidies remains to be the subject for further studies.

Conclusions

Across all hybrid types, breeding systems and localities, *P. esculentus* calls are predominantly shaped by the influence of the *lessonae*-genome (L) when it comes to the expression of advertisement call characteristics. However, several genetic particularities – such as genome dosage-sensitive expression in triploids, or more *P. ridibundus*-like call properties due to the “frozen” character of the clonal R genome in diploid systems - provide a perceptible fine tuning of hybrid call manifestation. As there is no rule without exception, we found that genome dosage-sensitive call patterns can be interrupted in certain populations, possibly due to random mutation, introgression or local selective forces.

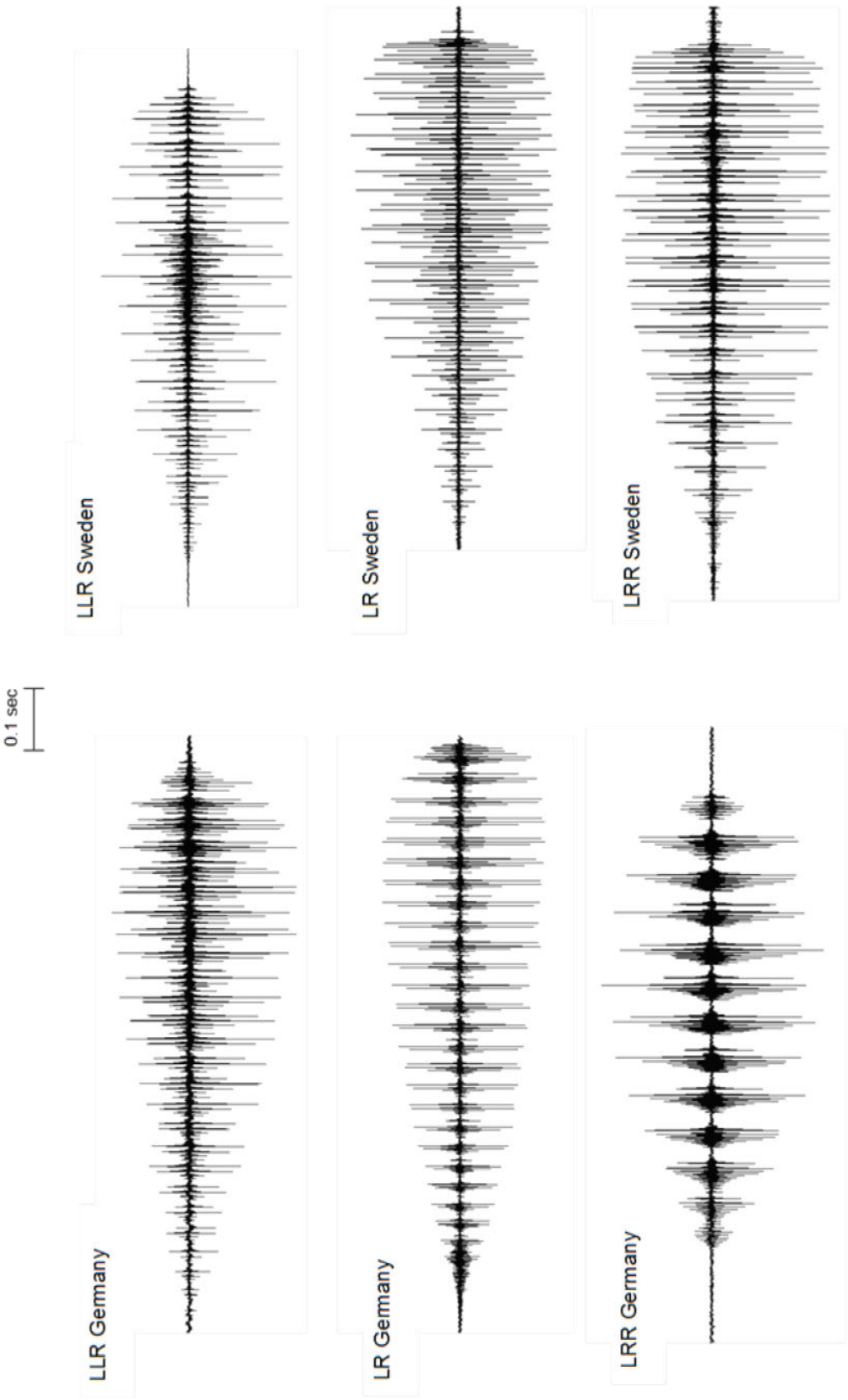


Figure 7. Comparative overview of advertisement calls from Germany (Döbern) and Sweden (Genarp). Shown are representative oscillograms of calls by each hybrid type recorded at approximately 20°C.

Although we can for now only speculate on the exact mechanisms behind the observed phenomena, our results most certainly confirm that *P. esculentus* populations are a genetically and phenotypically diverse clade. Apart from the evolution of reproductive strategies to gain independence from parental back-crossing, this group has developed regionally variable manifestations of genotype-dependent call differentiation, which could be related to several factors: 1) the reproductive modus responsible for the transmission of the R genome, 2) the features of the original parental genomic heritage and hybrid lineage, 3) the distance from the distribution edge of *P. esculentus* and its two parental species, and 4) the degree to which discrimination between genotypes plays a role in reproductive behaviors. Further studies and carefully designed mate-choice experiments could shed more light on the question whether pronounced call differences among males of different ploidies in most mixed ploidy populations are simply a neutral by-product of allopolyploidy, or could still have a reproductive function, e.g. to facilitate dissortative matings between diploid and triploid hybrids.

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Appendix 1. Microsatellite markers that were originally applied to genotype waterfrog individuals. Not all markers came to use for analysis in this study (see methods section). The column "dosage" indicates whether a marker could be used to identify triploid hybrids through dosage distribution in the L or R genome.

Locus	SequenceF	SequenceR	Ref.	GenBank No.	Use (this study)	Dosage
CA1b6	FAM - AAACTCGCGGTTTCCCTTAG	GAGCCAGGTTAAGATAACTGGAG	[75]	EF121548	L & R	yes
Ga1a19 redesigned	FAM - GAC TGG GAG GGA TAG GAA GG	CAG GGG ATT TTC CCA TCA G	[75]	EF121547	L & R	yes
Re1CAGA10	VIC - CAT GTT TAC CGT CAC TTT AAG AAC AC	CAT CTC TTC AGG TGG CTG GA	[75]	EF121549	R	No
Re2Caga3	NED - ATG TCG TTA GAG TTC ATA GG	ATC TCA AGT AAT CTG TCT GTC	[75]	EF121550	R	no
ReGA1a23	NED - ATT GCT TTG GCA GTG AAG G	TGA CAT CAC AGT GGG AGG AG	[58]	EU445523	L	no
Res16	FAM - GAT CCT GAT TTC CTG CT	GTT TAT TTA CTC TGT TCG TCT T	[104]	AF195843	not used	yes
Res20	VIC - TTT GTA AAT ATT CCG CTG GTA	CCG AGG TTG GCT GTC ATT A	[104]	AF195845	L	no
Res22	FAM - ATA CAG GGC TTA GTG AAA TGA A	AAG GGG TTA AAG GTG TGA CTA T	[104]	AF195846	R	no
RlCA18	FAM - CTC TGC TCC CTC AGC TAT GC	AAA AAG TGG TCC TTT CAT TTT GAG	[105]	AF286386	L	no
RlCA1a27	PET - GTT CAA GGG GGT CGA AAT AC	CAA ATG GGT CAT CCA CAC C	[58]	EU445522	L	no
RlCA1b5	NED - CCC AGT GAC AGT GAG TAC CG	CCC AAC TGG AGG ACC AAA AG	[105]	AF286388	L & R	yes
RlCA2a34	PET - GCT CCA TGC CAA AAG TCT TC	TTG GGT ATG ATA CTA CAA GCT ATG C	[58]	EU445521	L & R	no
RlCA5	VIC - CTT CCA CTT TGC CCA TCA AG	ATG TGT CGG CAG CTA TGT TC	[105]	AF286385	not used	no
Rrid013A	FAM - CGA GAA TCG AAG TGG AGA GG	ACC CGT CTC CAC AAT ACT GC	[106]	FJ024047	R	no
Rrid059A redesigned	NED - CCC CAT ACA TAT TGT TGG TTC C	ACA CTT ACA CTA AAA AGG ACA TTT ACC	[58]	FJ024048	R	no
Rrid064A	PET - TGT ACG GGC CTT TAG ACT GG	AAC TTT TTG AAG GCC CCT TG	[58]	EU445524	R	no
Rrid135A	NED - TCT TTT GTT TTA GCG CAC CT	CTG CCC GTC TAA GCA AGT GT	[58]	EU445526	R	no
Rrid169A	VIC - CGG AAC TCC GCT TTA ATC AC	CCC ATG TTG TCG TTG AGC TA	[58]	EU445525	R	no

Male spatial behavior and amplexus frequency in diploid and mixed-ploidy populations of water frogs: is there structuring by genotype?

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Abstract

Individual mating success depends on several genetic and ecological factors that affect, among other things, the size, behavior and spatial distribution of males and females. Here, we investigate the effects of these factors in populations of the edible frog *Pelophylax esculentus*, a natural hybrid between its parental species *P. ridibundus* (genotype RR) and *P. lessonae* (genotype LL), that can occur in different population systems. For this field study we observed edible frog populations in three geographically distant ponds: one pond in Switzerland contained diploid hybrid *P. esculentus* (genotype LR) and its parental species *P. lessonae* (thus the system is called diploid L-E system), the other two ponds (Germany and Sweden) contained only hybrids of diploid and triploid genotypes LR, LLR and LRR (thus called mixed-ploidy E-E system). Previous studies found that genotype can influence phenotypic characteristics in *P. esculentus*, and males of the parental species are considered to differ in their mating and spatial behavior during the reproductive season. To test whether genotypic differences among hybrid and parental males affected spatial behavior, we measured spatial parameters of males from the three ponds and tested them against genotype, body size, male condition and the frequency of observed amplexus by individual males. Our results yielded differences in spatial movement behavior among male water frogs, but these were not associated with genotype. Frogs from the Swedish mixed ploidy E-E system moved considerably larger distances between observations than did the frogs in the other two ponds. These differences were probably contributed to pond size and population density, which

was lower in the Swedish pond than in the other ponds. Spatial movement behavior by frogs in the remaining two populations (one a diploid L-E system, the other a mixed-ploidy E-E system) was the same. Furthermore, we did not any find any indication for genotype-related differences in spatial movement behavior and structuring between males in any of the observed populations. Males differed in body size between some genotypes, but only body size, not genotype, affected the distance between nearest male neighbors: larger males kept larger distances between them. The frequency of males observed in amplexus was neither affected by size, condition nor spatial behavior. In the two mixed-ploidy E-E populations, the genotype distribution of males observed in amplexus corresponded to the distribution of male genotypes in the population, thus indicating that no genotype had a mating advantage over another. In the diploid L-E population, *P. lessonae* had a tendency to prevail in amplexus frequency compared to *P. esculentus*, which is consistent with previous studies. Results are discussed with respect to the role of assortative mating in *P. esculentus* and the role of density in the study of mating systems

Introduction

Reproductive behavior plays a significant evolutionary role in the animal kingdom. Usually, specific reproductive behaviors are necessary for finding and assessing a suitable mating partner, and to avoid breeding with an unsuitable partner, e.g. an individual of a different species or one that is genetically incompatible. Especially when the reproductive period is strictly seasonal and individuals are otherwise not socially connected, specific behaviors and the right timing are important to recognize the right mating partner. While hybridization is usually avoided, some crossings between different vertebrate species resulted in viable hybrid systems that have evolved to be either reproductively independent from the parental species, or in the opposite way rely on back-crossing with parental genotypes to perpetuate their genes to the next generation. Anurans are a group of vertebrates which have evolved a number of successful interspecific hybrids and exhibit a variety of mating systems that are most commonly shaped by female choice and male-male competition (Emlen and Oring 1977b, Arak 1983). This makes this group of amphibians an ideal system to study mating behaviors of hybrids and their parental species.

A prominent example of a successful and widely distributed interspecific anuran hybrid is the European water frog *Pelophylax esculentus*. *P. esculentus* is a natural hybrid between the pool frog *P. lessonae* (genotype LL) and the marsh frog, *P. ridibundus* (genotype RR). Despite the fact that *P. esculentus* is one of the most common amphibians in Europe, its hybrid nature was not known until the 1960s, when Berger (1967) discovered it through crossing experiments. *P. esculentus* comes in different reproductive modes, which are described in Graf and Polls Pelaz (1989). Early studies found that *P. esculentus* reproduces through hybridogenesis (Berger 1968, 1970, Tunner 1974), a hemiclonal reproductive mode first described in fishes of the genus *Poeciliopsis* by Schultz (1969): Hybrids exclude the genome of one parental species prior to meiosis and transfer the genome of the other parental species clonally to the next generation, i.e. without recombination (Tunner 1974, Tunner and Heppich-Tunner 1991, Zalesna et al. 2011). In order to restore hybridity in their offspring, hybrid males and females have to back-cross with the parental species whose genome is excluded. In areas, where *P. esculentus* occurs sympatrically with *P. lessonae*, the L genome is excluded and regained through matings between the hybrid and *P. lessonae*. This is called the L-E-system (Graf and

Polls Pelaz 1989). In other areas, a mirror system exists where the R genome is excluded and hybrids back-cross with sympatric *P. ridibundus* to regain it. In both systems, the hybrid is a sexual parasite that relies on a parental species (the sexual host) for successful reproduction; and in both systems the resulting offspring are diploid *P. esculentus* (genotype LR). Since these hybrids do not recombine their two genomes, recombination can only take place between LL and RR in the parental species *P. lessonae* and *P. ridibundus,* respectively. A different situation exists in all-hybrid populations consisting of diploid (LR) and triploid hybrids (LLR and/or LRR). These so-called E-E-systems occur mainly in northern Europe around the Baltic Sea, but also in some areas of eastern and Central Europe. Here, hybrids have become reproductively independent of the parental species as a result of polyploidization and "meiotic hybridogenesis" (Alves et al. 2001). This means that triploids of both sexes premeiotically exclude the genome present in one copy (R in LLR and L in LRR), recombine the double genome and transfer it to haploid gametes (L in LLR and R in LRR), as the parental species *P. lessonae* and *P. ridibundus* do in L-E- and R-E-systems, respectively. When these haploid gametes fuse with diploid ones (LR) that are usually produced by diploid females, triploid offspring result; when they fuse with heterospecific haploid gametes produced by other triploids or by diploid LR (usually males) diploid offspring arise (Günther et al. 1979, Christiansen et al. 2005, Arioli 2007, Christiansen 2009, Christiansen and Reyer 2009). As a result, both hybrid types are mutually dependent on each other: triploids are the sexual hosts for diploid sexual parasites and vice versa (Som and Reyer 2006).

The two parental species (LL, RR) and the different hybrid types (LLR, LR, LRR) differ in several features, including morphology, ecology and behavior (Blankenhorn 1974, Berger 1977, Günther 1990, Plötner 2005, Jakob et al. 2010, Embrechts and Reyer 2012). Relevant for the present study are potential differences between genotypes in mating behavior. Both parental species are prolonged breeders sensu Wells (1977), i.e. their breeding season spans several weeks to a few months between April and July, with a reproductive peak between mid-May to June (Günther 1990, Plötner 2005). Most mating systems of prolonged anuran breeders can be classified as types of resource defense polygyny (males control access to females by monopolizing resources used by females), male dominance polygyny (males aggregate and display during the reproductive season and are selected by females) or lek systems, where males do not defend resources but

directly compete for a dominant status or the best position within an assembly of males frequented by females in search of a partner (Emlen and Oring 1977a, Wells 1977, Arak 1983, Wells 2007). In the case of *P. ridibundus*, the mating system has been described as a form of resource defense polygyny, because males defend small areas that provide access to females and/or to resources used by females, such as oviposition sites (Kuhn and Schneider 1984, Kyriakopoulou-Sklavounou and Loumbourdis 1990). In a mixed system with both *P. esculentus* and *P. lessonae*, *P. ridibundus* males have been reported to take advantage of their bigger size to aggressively drive the smaller *P. lessonae* males away from spawning sites (Lada et al. 1995). However, the authors emphasize that the aggressiveness by *P. ridibundus* males gradually decreased over the breeding season, while it increased in males of the remaining two genotypes. Findings from other studies indicate that the strict assignment of *P. ridibundus* to resource defense polygyny might not be unambiguous, since physical defense of territories was rarely observed (Tunner 1976, Weidenberg 1999) and territory sizes varied highly between individuals (Günther 1990, Fischer 1996). Rather than engaging in physical interactions, as it is common in extremely territorial species like male bullfrogs (*Rana catesbeiana*, Howard (1978)), *P. ridibundus* males respond to conspecific calls by increasing the intensity of their advertisement calls, which, in a mixed chorus, are comparatively louder than those of *P. lessonae* or *P. esculentus* (Brzoska 1982). Regarding male reproductive behavior in *P. lessonae,* literature sources commonly agree that males do not defend extensive territories during the mating season, but move around within large choruses to intercept and clasp females (Blankenhorn 1974, Tunner 1976, Lengagne et al. 2008). Although comparative studies on mating behavior between the two species are scarce, the spatial movement behavior of *P. ridibundus* and *P. lessonae* males appears to differ in so far, that the tendency in *P. ridibundus* goes more towards spatial site tenure and in *P. lessonae* more towards spatial roaming behavior (Lengagne et al. 2008).

Due to the hybrid nature of *P. esculentus*, several studies support the hypothesis that the hybrid takes an intermediate position in reproductive behavior between the two parental species. When compared to *P. lessonae* in terms of mating speed and chorusing behavior, hybrid males are less active and more stationary than *P. lessonae* (Lengagne et al. 2006, Lengagne et al. 2008). When directly compared to *P. ridibundus* , *P. esculentus was* observed more often in amplexus and showed

higher sexual activity than *P. ridibundus*, although there appears to be no difference in calling activity, agonistic behavior or territory tenure (Weidenberg 1999). Hybrids therefore seem to share the tendency to be stationary at territories with *P. ridibundus*, but due to their *P. lessonae* heritage, are also active in intercepting and clasping females. In this study we were interested whether a gradient in these behaviors (stationary versus roaming and high versus low events of amplexus) exists among hybrids of different hybrid systems, and whether this gradient is influenced by the hybrid genotype.

So far, most behavioral studies of *P. esculentus* have focused on diploid hybrids (genotype LR) in which the ratio between the two parental genomes is 1:1. In triploid LLR, however, the L/R ratio is 2:1 and in LRR it is 1:2. Because of this dosage effect, LLR triploids can be expected to resemble more *P. lessonae*, while triploid LRR should resemble more *P. ridibundus.* Such genomic dosage effects have indeed been found for phenotypic characteristics of *P. esculentus* like body size (Embrechts and Reyer 2012) and morphology (Ebendal 1979, Ebendal and Uzzell 1982, Plötner et al. 1994, Kierzkowski et al. 2011). Whether dosage also affects reproductive behaviors in this hybrid complex has been largely unknown because with rare exceptions (Günther and Plötner 1990), reproductive behavior in populations with polyploidy hybrids was not studied. Only recently it was found out that advertisement calls of male hybrids are not only intermediate between calls of parental males, but calls of LLR, LR and LRR also differ from each other as expected from genomic dosage effects (Hoffmann and Reyer 2013). In other polyploid anurans, it has been shown that differences in ploidy can alter male calling behavior (and even the corresponding female preferences) which has been demonstrated for the two cryptic North American treefrog species *Hyla chryoscelis* (diploid) and *H. versicolor* (tetraploid), which differ in ploidy (Gerhardt 2005a, b).

In the present study we examined whether the reproductive strategies of hybrids in terms of space use (territorial versus roaming) differ between genotypes and whether they show signs of a genomic dosage effect. Since males that move around more extensively within a pond should encounter more possibilities to clasp a female (Wells 1977), we tested for any effects of spatial movement behavior on male mating success by relating space use parameters to observed amplexus of individual males. We performed our study in three different ponds, one in Switzerland with an L-E

population and two in Germany and Sweden with E-E populations. Specifically, we addressed the following questions:

1. Do the distances males move between observations and the distribution of their overall home ranges differ between individuals with different genomic composition?
2. Do the distances and ranges correlate with body size and/or condition?
3. Do nearest neighbor distances vary between genotypes?
4. Is the frequency of amplexus events affected by genome composition and/or male characteristics like space use, body size and body condition?

Given that previous studies had demonstrated the intermediate nature of hybrids in several characteristics, we expected that triploids should behaviorally tend more towards the parental species they share two genomes with; i.e. LLR males should move around more than LRR individuals, and LRR males should be more stationary than LLR individuals, whereas LR individuals should be intermediate between the two types of triploids. To test this hypothesis, we compared the reproductive success and spatial behavior of genotypes during the reproductive period. Reproductive success was measured by amplexus frequency, space use by regularly recording the positions of marked individuals. As an indicator of spatial tenure we measured the distance an individual male had moved between subsequent transect observations and the distance to the center of its home range.

Methods

Sampling periods and sites

Field data for this study were collected at three natural ponds located in Northern Switzerland (Kloten), Eastern Germany (Döbern) and Southern Sweden (Genarp). These three ponds were situated more than 500km apart (Figure 1). In Döbern, catching, marking and transect sampling took place between 30th April to 2nd June 2009 and in Genarp between 4th and 26th June the same year. In Kloten, where data were originally collected in the scope of a different study (Abt Tietje 2003), the corresponding field work was performed in 1992 from 4th May until the end of August. For the present study, however, only data from May and June 1992 were used to cover the same seasonal period for all three ponds. Due to the deviating objectives,

some sampling methods did also differ between Döbern/Genarp in 2009 and Kloten in 1992 and are therefore described separately, where necessary.

Figure 1: Map showing the localities of the three ponds Kloten (Switzerland), Döbern (Germany) and Genarp (Sweden).

Capturing, measuring and marking of frogs

At all three ponds, frogs were captured by hand at night using flashlights and kept in cool and moist boxes until the next morning. Then they were measured (snout-vent length, SVL) to the nearest mm using calipers and weighed to the nearest 0.5 g with a spring balance. From SVL and body mass (BM) we later calculated a body condition index (BCI) according to the equation $BCI = BM/SVL^3$ (Jakob et al. 1996) . After measuring, each frog was marked with an individually numbered tag that was fitted around the frog's waist with dental floss (1992) and rubber thread (2009), respectively. We made sure to fit the bands loosely enough to allow for normal movement, including oviposition in females. In 1992, we re-caught the frogs at the

end of the season and removed the waistbands, whereas the thread used the in 2009 was degradable and fell off by itself after several weeks, i.e. after the observation period. In 2009, we also took photographs of the dorsal and ventral pattern, in case animals needed to be identified if their tag got lost or became unreadable. This turned out to be the case for two males, which could then both be identified by their dorsal pattern. We marked only adult frogs > 45 mm that could be reliably sexed by the presence (males) or absence (females) of vocal sacs and thumb pads.

For determining genotypes we took lymph from a small incision into the foot web (1992), respectively two toe clips from the 1st phalanx of the 2nd digit of the front legs (2009). The clips were stored in 70% ethanol, and from the wound a blood smear was produced on a microscopic slide. As taking toe clips and blood smears for genotype determination was more invasive than taking lymph, we attempted to minimize stress as much as possible. Therefore, all frogs sampled in 2009 were anaesthetized before handling by bathing them for 10-30 minutes in a diluted and buffered MS222 solution (Mitchell 2009). After handling, they were stored again in a moist and cool lidded box and given 2-3 hours to rest and recover, before they were released back into the pond. Thereafter, they were observed for a while to make sure they resumed normal activity, which all of them did (e.g. hopping directly towards the water, swimming towards floating vegetation, calling). We did not observe any physical impairment or deaths as a consequence of handling in any of the three ponds.

Genotype determination

In 1992, genotypes in the Kloten population were determined through protein electrophoresis of lymph samples, following standard procedures (e.g.Uzzell and Berger 1975, Uzzell and Hotz 1979). In 2009 genotype determination for Döbern and Genarp was done in two complementing ways. First, the largest length and width of erythrocytes were measured from blood smears using an optical microscope with 400 x magnification. Measurements were taken from at least 6 cells per individual, which were randomly chosen among cells of representative size and typical ellipsoid shape. Erythrocyte size was then calculated from an ellipsoid equation using the average of the multiple measurements for each individual. Erythrocyte planimetry is a fast and reliable method to distinguish between diploid and triploid frogs in the field,

but it does neither allow to tell triploids of different genome composition apart (i.e. LLR and LRR), nor does it give unambiguous results in separating diploid LR hybrids from the two parental species LL and LR (Schmeller et al. 2001, Jakob 2007 ch. 1). As a better, but more time consuming, separation of genotypes we therefore performed microsatellite analysis on DNA extracted from the toe clips. Of the 16 markers that we used, four are specific for the L genome, eight for the R and four amplify in both genomes with a dosage effect. This marker combination allows unambiguous identification of all genotypes, including distinction between LLR and LRR hybrids. Details of the markers and the lab techniques have been described in Chapter 1-3 of this thesis and by Christiansen (2005, 2009), Christiansen and Reyer (2009), Arioli et al. (2010), Jakob et al. (2010).

Pond features and population density

We surveyed and sketched the ponds true to scale, including details on the type and extent of the vegetation. In 1992, we measured the Kloten pond size from this sketch, whereas in 2009 the pond areas for Döbern and Genarp were obtained from aerial pictures using the measuring feature in program Quantum GIS (QGIS Development Team 2013). Pond sizes were rounded to the nearest m^2. To estimate population size and density, different methods had to be used for the two years of the study. In 1992 in Kloten we recorded only marked individuals during our daily observations (see below), but we caught animals repeatedly through the whole season. This capture-mark-recapture design allowed calculation of the population size through the Jolly-Seber method (Caughley 1980). In contrast, frogs in the Döbern and Genarp ponds frogs were, once marked, not captured again, except if they had lost their tag. Thus, the assumption of the Jolly-Seber method, that marked and unmarked animals are equally vulnerable to capture, was not met in these populations. The capture data from Döbern and Genarp corresponded more to a depletion capture method, since we captured and marked as many frogs as possible at the beginning of the season during repeated captures. The assumptions of this method (e.g. relatively small capture area, negligible amounts of emigration during the sampling period, all frogs equally vulnerable to capture) were met in both ponds. We thus used the capture data from Döbern and Genarp to estimate population size based on depletion capture counts as described by Zippin (1958). Densities were calculated by dividing the estimated population sizes by the respective pond size.

Data collection

Data were collected by slowly walking along the edge of a pond, recording locations of the frogs and entering them into a detailed map of the pond. Positions could be determined quite precisely through a combination of prominent vegetation in the pond, natural features of its bank and sticks that we had placed as artificial landmarks along its edge. In Kloten we recorded only marked individuals, in Döbern and Genarp, unmarked frogs were recorded as well, but genotypes (and sometimes sexes) were not securely distinguishable. In accordance with previous studies on hybrid *P. esculentus*, frogs < 45 mm body length and no perceptible sexual characteristics were recorded as "juvenile" (Arioli 2007, Christiansen and Reyer 2009). In all three ponds, marked males in amplexus were documented whenever observed. If an amplexus pair was encountered and the male was not marked, we tried to capture the male to determine its genotype, measure its size and weight and to mark it individually for later observation.

With the exception of cold and rainy days when there was no calling activity, observation rounds were done daily 1-3 times during daytime over the periods given in Table 1. To guarantee independence of the data, we allowed for a minimum of 5 hours between successive observations. Since mating activity of frogs is strongly temperature dependent, we measured water temperature continuously. In Döbern and Genarp, we placed three thermocouples each 15 cm below the surface and recorded and stored temperature every 30 minutes on three temperature loggers. The mean temperature across the three loggers ranged similarly between 12° and 25°C (Döbern, early May-early June) and 10° and 20°C (Genarp, early-late June), with fluctuations between day and nighttime and periods of warmer versus colder weather. In Kloten, temperature was not measured in 1992, but continuous logger recordings from 1994-1996 in the same pond showed that in all three years water temperatures during peak reproductive activities were very similar, ranging from about 12°C in early May to around 20°C in mid-June. Therefore, we believe that these temperatures are also representative for 1992. During our observations, water temperature was > 15°C and thus favorable for mating activities (Wahl 1969, Blankenhorn 1974, Heym 1974).

Calculation of spatial data

The observation sheets with the frog locations were digitized with geographical information system (GIS) software (Quantum GIS in 2009). Coordinates of all recorded frogs were saved as point vector layers. These point layers were imported into spreadsheet format to sort the data by individuals and link them with genotype, sex, size, weight and body condition. From the location data of marked individuals we derived three spatial measurements:

1. Distance between observations (DBO): For each individual that was observed more than three times, we calculated the Euclidean distance between two subsequent observations. These distance measurements were then averaged across the number of total observations per individual to get a measure for the spatial activity.
2. Distance to center (DTC): For each individual with more than three observations, the distance of each recorded location to the center of the activity range (=centroid) was calculated. Although to some extent correlated with DOC, this variable provides additional information whether a frog's movements were condensed in a certain area or spread out over a larger area. Hence, it is an approximation of "territory size"
3. Distance to nearest neighbor (DNN): To measure proximity to the nearest neighbor, we averaged the distance of a given male to its closest male neighbor across all occasions the focal individual was observed (minimum of 3 observations).

Based on our observations, we considered only nearest-neighbor distances of > 3m relevant for physical male-male interactions. In the field, we did not observe any physical interactions between males that were more than 3m apart, and only in 2.76 % of 1277 nearest-neighbor distances the observed male individuals kept more than 3m between themselves and the nearest male. Most of the nearest-male distances > 3m were measured in Genarp and Kloten. In Genarp we assumed that the larger distances (max. 19m) of some males to their nearest male neighbor could be attributed to the generally low population density and we further assumed that males would not interact physically at such distances. For the Kloten outliers, we assumed that they could be caused by the less stringent sampling regime which did not account for unmarked individuals. We thus felt confident that omitting all cases of

distances > 3m between males provides a justified correction of the data for further analysis. Since we tested for the influence of genotype and morphological characteristics on nearest neighbor distance, we only considered cases where the genotype and body measurements of both neighbors where known. During an observation round, each individual neighbor pairing was only recorded once and thus pseudoreplication was avoided.

Statistical analysis

All statistical tests were performed using program Systat (version 11). To compare the three distance measures DBO, DTC and DNN between and within populations, genotypes, sexes and relate them to body size and condition, we performed generalized linear models (GLM). In the analysis of DNN, we incorporated body size and condition as a) the averaged values between the two nearest neighbors (AV_SVL and AV_BCI) and b) the difference between these two values (DIFF_SVL and DIFF-BCI). Unless otherwise stated, probability levels below $\alpha = 0.05$ were considered significant. For Post-hoc tests we used Fisher's least significant difference test with subsequent Bonferroni correction. When plotting centroid coordinates of individuals from a pond onto a map, we calculated Kernel-density estimates using the in-built function in Systat and choosing the default probability level of 68%. The observed distribution of genotypes among amplexus males was tested against the expected distribution of genotypes among males in the population using Chi^2-tests.

Results

Pond features and population composition

The three study ponds differed in several features (Table 1). With a surface area of 90 m^2, Kloten was by far the smallest pond, followed by Döbern (537 m^2). The Genarp pond (3352 m^2) was about 37 times larger than the Kloten pond and six times larger than the Döbern pond. In Kloten about 45 % of the surface area was open water, 25% was covered by water lilies (*Nuphar lutea*) and 30% consisted of sedge regions (*Carex spec.)* (Figure 2A). The pond was surrounded by bushes and small trees which were too low to cast shade onto the water, except during the very early and late hours of the day. In Döbern, 25% of the surface area was open water, 20% was covered by water lilies and 35% by reeds of medium to low density (Figure 2B). The pond surface was further broken by two small, densely vegetated islands that covered 20% of the total area. Vegetation on the islands and surrounding the pond cast little shade onto the water, and only early and late in the day. At the Genarp pond, open water areas comprised 80% of the surface area and the edges were covered with high reeds (12%) and sedges (8%, Figure 2C). In the center of the open water area there was a coherent patch (about 10% of surface area) of submerged water vegetation that reached close to the water surface and was used by the frogs for spawning. Like the other two ponds, the Genarp pond was hardly shaded by surrounding vegetation.

Table 1: Comparative overview of sampling and pond details of the three sampled localities. Sample sizes are given per genotype and sex.

	Kloten	Döbern	Genarp
Sampling period	15 May – 20 June 1992	24 May - 2 June 2009	13 - 26 June 2009
Total number of observations	37	14	12
Pond size	90 m^2	537 m^2	3352 m^2
Population size	approx. 350	approx. 260	approx.560
Density	3.9 frogs/m^2	0.48 frogs/m^2	0.15 frogs/m^2
Marked individuals in analysis:			
LL	M: 97 / F: 67		
LR	M: 14 / F: 63	M: 15 / F: 6	M: 31 / F: 1
LLR		M: 17 / F: 2	
LRR		M: 25 / F: 5	M: 1 / F: 1

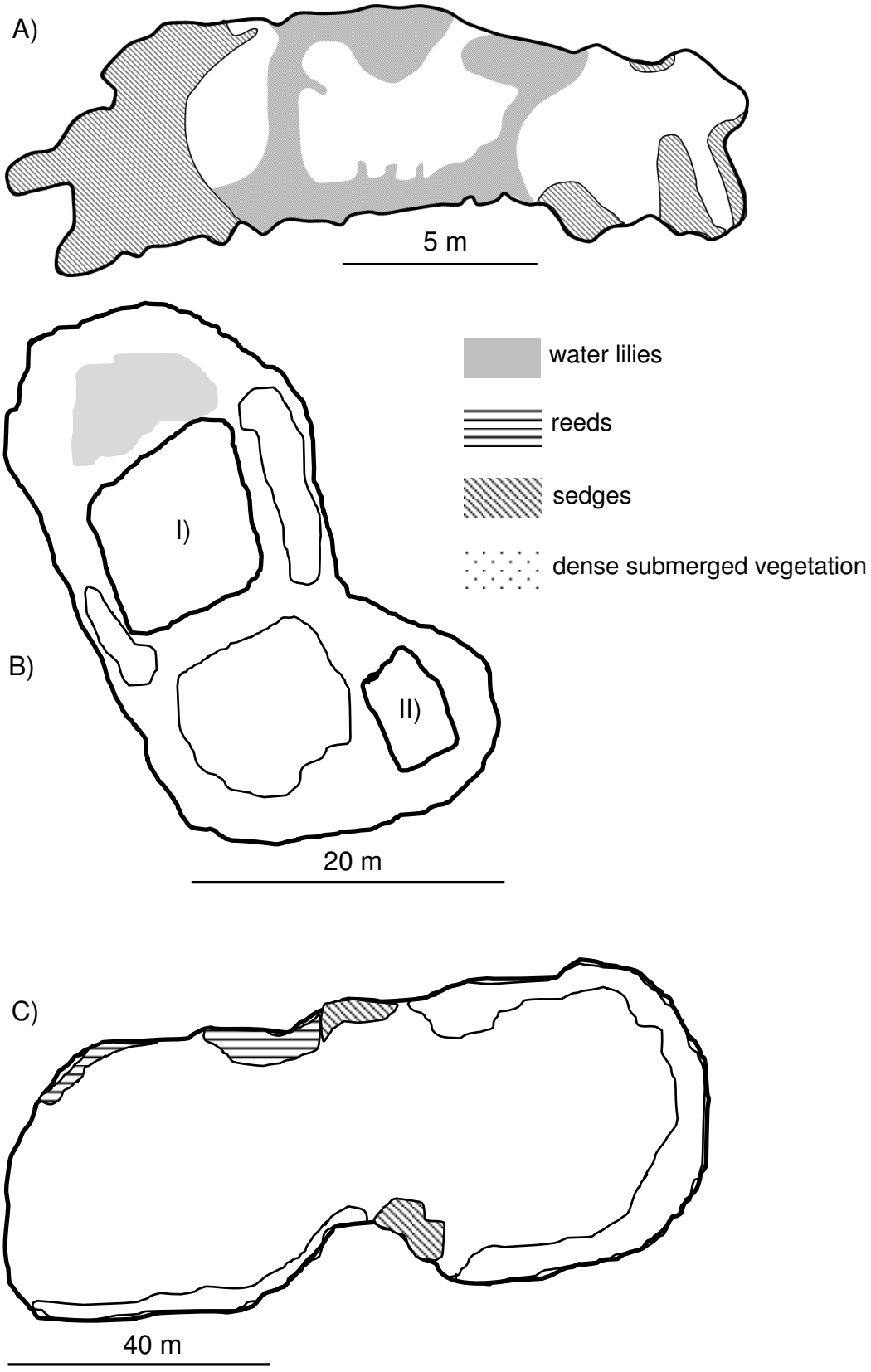

Figure 2: Schematic illustration of the three study ponds A) Kloten, B) Döbern and C) Genarp, with distribution of open water and predominant vegetation types. I) and II) indicate vegetated islands in the Döbern pond.

The absolute number of frogs was highest in Genarp and slightly lower in Döbern and Kloten. However, due to the different pond sizes, densities per m^2 increased in the order Genarp – Döbern - Kloten (Table 1). In Kloten, 70% of the individuals were *P. lessonae* with a male/female ratio of 1.26, 28% were *P. esculentus* of LR genotype (sex ratio 0.23), and 2% were *P. ridibundus* (females only). The Döbern and Genarp populations consisted to 100% of *P. esculentus* genotypes. In Döbern, the male/female ratio in 2009 was 1.44 and showed sex differences in genotype composition. Most females in Döbern were diploid LR (51%), followed by LRR (41%) and LLR (8%). Among males, triploid hybrids together comprised the majority of individuals (77%). LRR males were the most numerous in the population (48%) followed by LLR (29%) and LR males (23%). In Genarp, the male/female ratio was highest with 2.01. The distribution of genotypes among females was similar to Döbern (LR: 47%, LRR: 47%, LLR: 8%), but among males we observed a different pattern. The vast majority of males were diploid LR (86%). LLR triploids comprised a smaller part (11%), and LRR males were markedly rare in the population (3%).

Frog characteristics

In two GLM analyses, we tested several population parameters for differences in body size (SVL) and body condition index (BCI). Both size and condition index differed strongly between populations, genotypes and sexes (Table 2). Furthermore, an interaction between sex and genotype significantly affected SVL, but not body condition.

Table 2: GLM of body size (SVL) and body condition index (BCI) versus population, genotype and sex. Significant p-values are printed in bold.

	SVL			BCI		
Source	df	F-ratio	P	df	F-ratio	P
population	2	20.477	**< 0.0001**	2	11.166	**<0.0001**
genotype	3	24.91	**< 0.0001**	3	4.001	**0.008**
sex	1	12.546	**< 0.0001**	1	7.035	**0.008**
sex*genotype	3	2.917	**0.034**	-	0.677	0.566
error	335			338		

Post hoc tests showed that all three populations significantly differed in average body size (Kloten-Döbern: $p = 0.006$, Kloten-Genarp: $p < 0.0001$, Döbern-Genarp: $p < 0.0001$; Bonferroni-corrected $\alpha = 0.0167$). Average body sizes were largest in Döbern, second largest in Genarp and smallest in Kloten (Fig. 2A). For the variable BCI, only differences between Kloten (highest average values of BCI, Fig. 2B) and Döbern, and between Kloten and Genarp were significant (Kloten-Döbern: $p = 0.017$, Kloten-Genarp: $p < 0.0001$, Döbern-Genarp: $p = 0.073$; Bonferroni-corrected $\alpha = 0.0167$). Females were, on average, 5.05mm larger than males ($p < 0.0001$). However, only size differences between LL (males and females) versus most other genotypes of both sexes were significant after Bonferroni-correction (Table 3), as were differences between LLR females versus males of the LLR and LR (but nor LRR) genotype. Among the rest of genotype combinations, there was too much overlap in size to yield statistically robust differences. Yet, there was a tendency of LRR males to be larger than LLR and LR males (Figure 3A, Table 3

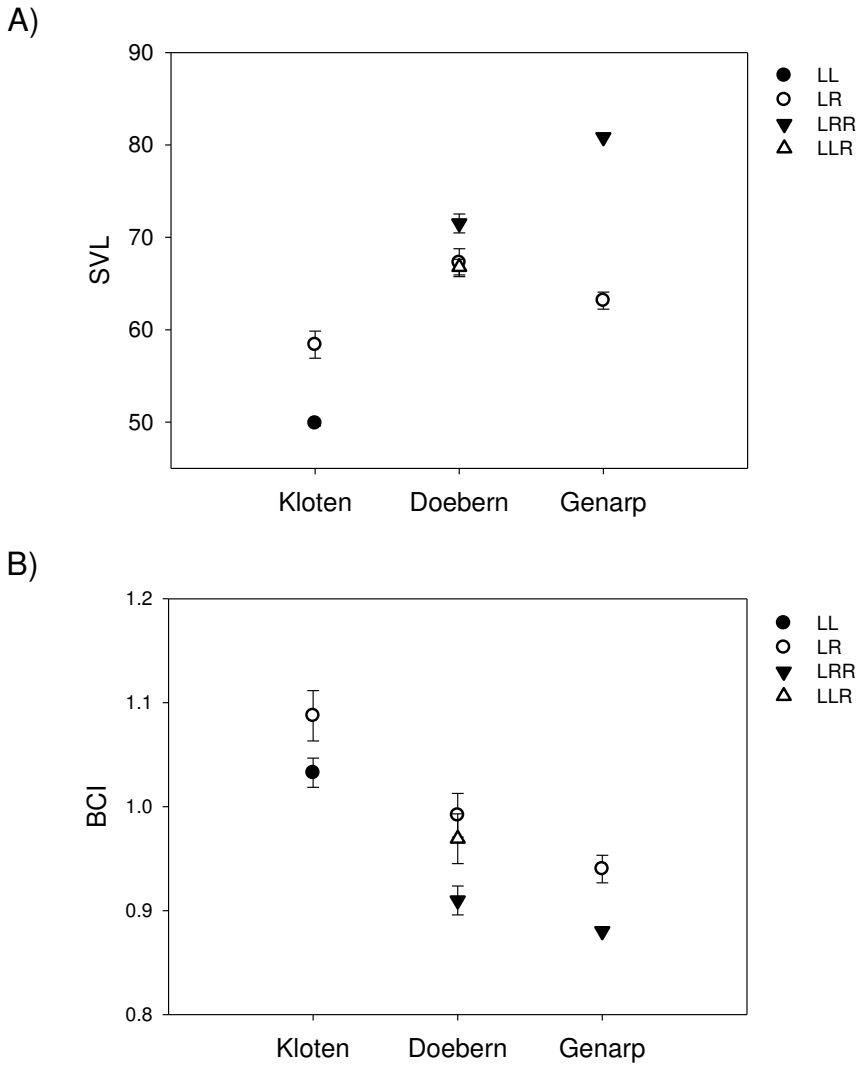

Figure 3: Male body characteristics by genotype for the three ponds. SVL in (A) represents male body size ("snout-vent length") in mm. BCI in (B) means body condition index and was calculated from body size and weight and gives an indication of a males' nutritional condition (see methods). Symbols indicate group means, and error bars ± 1 S.E.

Table 3: Matrix of pairwise comparison probabilities of SVL differences after Fisher's least significant difference test (Bonferroni-corrected α = 0.00625). Numbers indicate p-values, significant values are printed in bold.

	LL-F	LL-M	LLR-F	LLR-M	LR-F	LR-M	LRR-F	LRR-M
LL-F	1.000							
LL-M	0.2810	1.000						
LLR-F	**< 0.0001**	**< 0.0001**	1.000					
LLR-M	0.024	**0.006**	**0.002**	1.000				
LR-F	**< 0.0001**	**< 0.0001**	0.018	0.103	1.000			
LR-M	**< 0.0001**	**< 0.0001**	**0.003**	0.628	0.059	1.000		
LRR-F	**< 0.0001**	**< 0.0001**	0.125	0.019	0.265	0.030	1.000	
LRR-M	**< 0.0001**	**< 0.0001**	0.039	0.008	0.425	0.015	0.561	1.000

For BCI, we did not identify any interactions between sex and genotype. Rather, differences between the sexes were significant across all genotypes with females showing BCI values of 0.04 units lower than males (p=0.008). According to pairwise genotype comparison, BCI differed significantly between the LR and LRR (p = 0.013, Bonferroni-corrected α = 0.0125), but not among the rest of genotypes (Figure 3B).

Spatial distribution and movements

The mean distance between two subsequent observations of the same frog (DBO) ranged from 0.45 to 7.43 m in Döbern (n = 61), 0.39 to 8.71m in Kloten (n = 229) and 6.35 to 16.74 m in Genarp (n = 32). We entered population, sex, genotype, body size (SVL), body condition (BCI) and the interaction between sex and genotype into a GLM on distance parameters DBO (mean distance between subsequent observations) and DTC (mean distance to the centroid of all observations). Results are shown in Table 4. Across all sexes and genotypes, DBO and DTC were larger in Genarp than in the other two populations (Figure 4A-B), thus yielding a strong significant effect in the model (Table 4). Sex, genotype, size, body condition and the interaction between sex and genotype did not contribute to either the model for DBO nor DTC and were thus eliminated during step-wise variable selection (Table 4).

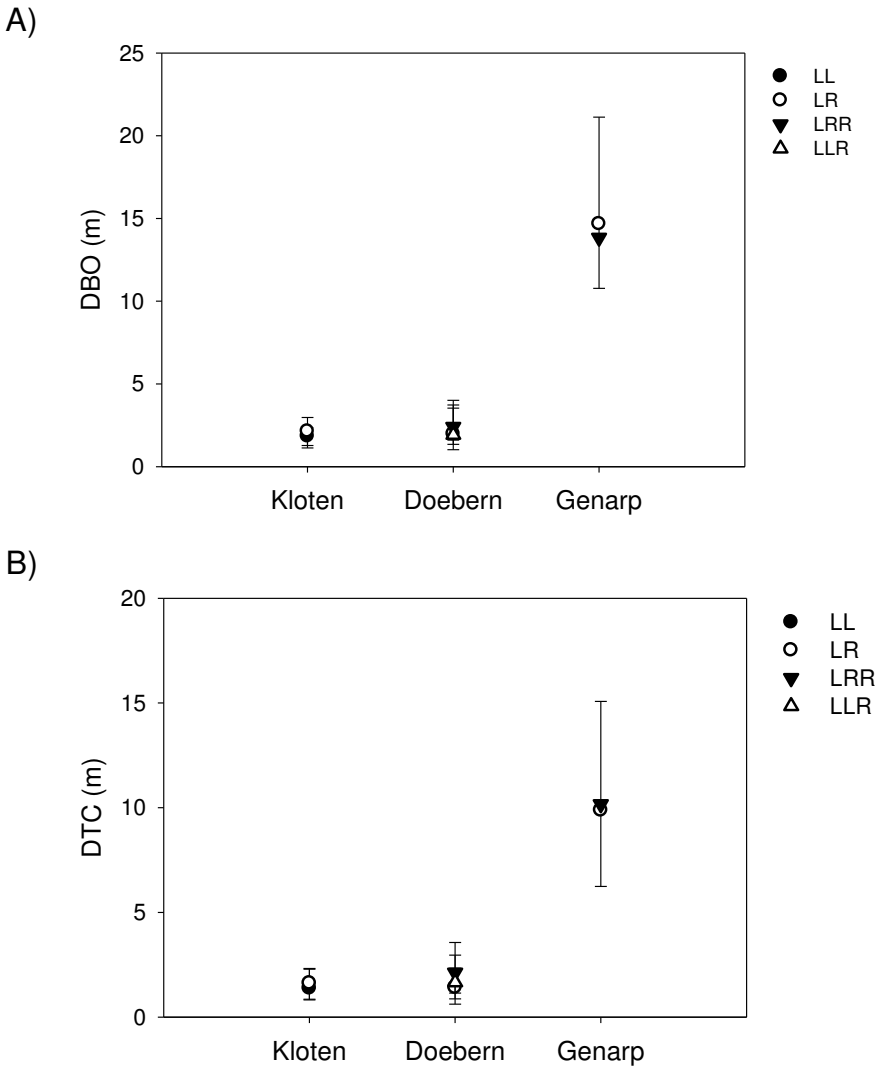

Figure 4: Distance measurements by population and genotype. Symbols indicate group medians, error bars indicate 75%- and 25%-percentiles.

Table 4: GLM of movement distance parameters (DBO and DTC) versus population, genotype and sex. In the 'df'-column, (-) indicates variables that were eliminated during step-wise variable selection due to non-sufficient p-values. Significant p-values are printed in bold.

	DBO			DTC		
Source	df	F-ratio	P	df	F-ratio	P
population	2	417.052	**<0.0001**	2	214.213	**<0.0001**
sex	-	2.283	0.132	-	1.674	0.197
genotype	-	0.443	0.722	-	0.455	0.714
SVL	-	1.035	0.31	-	0.777	0.379
BCI	-	0.022	0.881	-	0.385	0.535
sex*genotype	-	0.18	0.91	-	0.165	0.92
error	349			349		

To illustrate the location of the individual movement centroids as obtained by individual position data, we plotted the xy-coordinates of the centroids onto maps of the three ponds. The areas of these Kernel estimates overlapped greatly among the sexes and genotypes, thus not yielding any indication of sex- or genotype-specific spatial structuring (Figure 5A-D). The kernels covered mainly the right half and the center part of the pond, while less movement centroids fell into the left half of the pond. This part of the pond that was apparently less frequented by the frogs corresponds to a coherent patch of dense vegetation (sedges, see Figure 2). In the Döbern pond, we also found a great overlap among the kernels of LLR, LR and LRR males (Figure 6A). Since females were recorded at a lesser sample size, all genotypes were pooled into one graph, which also shows no pattern in the distribution of female movement centroids (Figure 6D). For the Döbern one should not forget the two islands in the pond (Figure 2B), which are not shown on the maps in Figure 6A-D. An aggregation of centroids in the center of the pond and in the right lower half of the pond across all genotypes does not mean that frogs actually moved around on or travelled terrestrially across the islands. In fact, we only observed frogs on land by the very edges of the islands. The edges of the islands were attractive spots that were frequented by a large number of frogs. This was especially the case for LRR males, which yielded two kernel areas that overlap with the location of the islands I) and II) in Figure 2B. In the Genarp pond, only LR males could be tracked in sufficient numbers to obtain movement centroids and estimate kernel ranges from their distribution (Figure 6E). Interestingly, the position of the kernel, which includes most centroid locations, is situated in the center of the pond, which at the same time is the narrowest part between two pond banks. Figure 2C shows that this part of the pond is overgrown by a large and dense patch of submerged water vegetation, which reached close to the water surface. During our observations, we saw many frogs using the pond banks for basking and calling, which they also did within dense vegetation like reeds and sedges. However, the patch of water vegetation was a preferred spot for oviposition. Amplexus pairs moved there for spawning, and during times of high calling activity, males frequented the area in search of unpaired females.

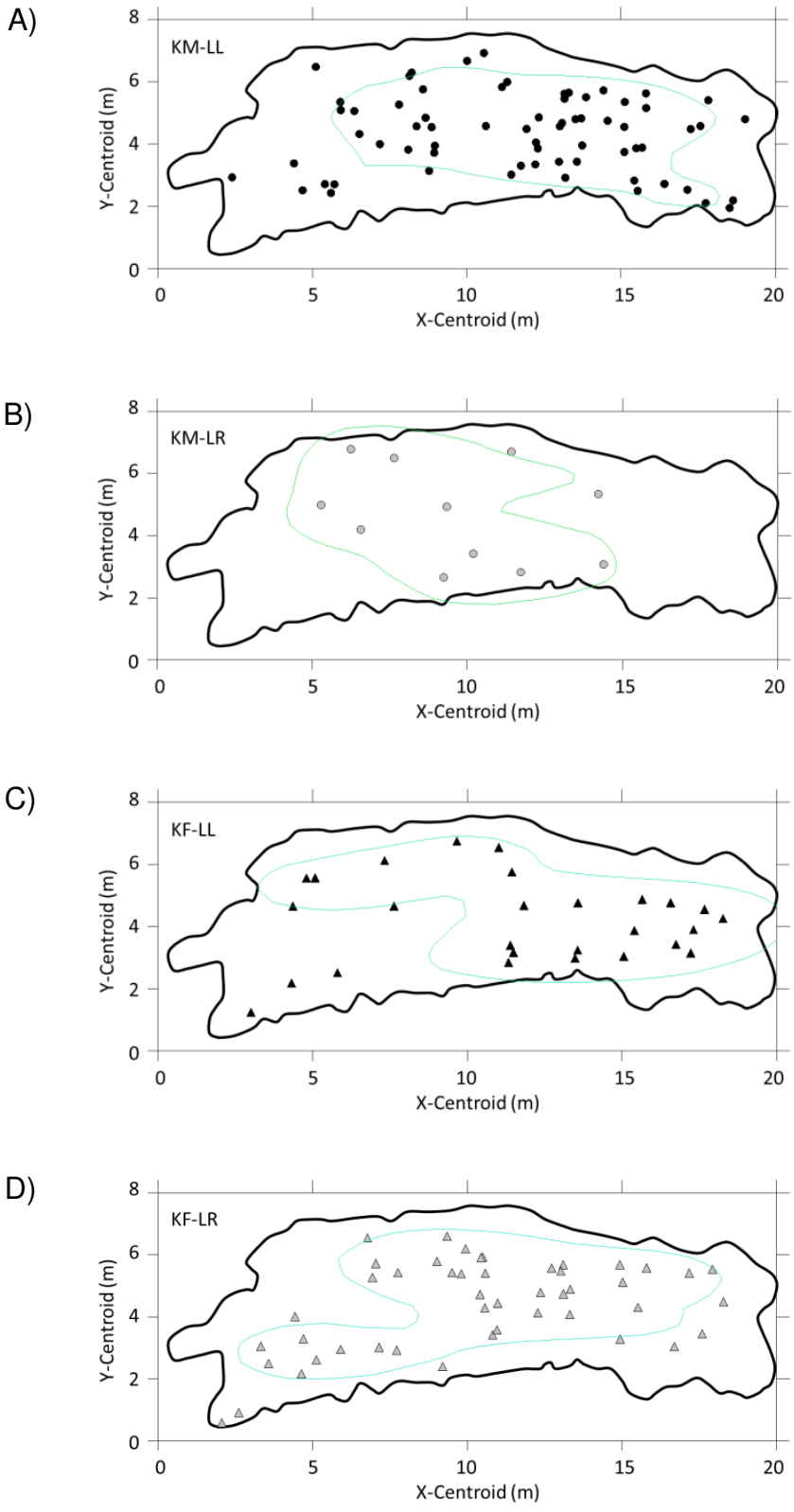

Figure 5: Distribution of centroids for males (A-B) and females (C-D) of *P. lessonae* (LL) and *P. esculentus* (LR) in Kloten. Green lines indicate 68%-Kernel estimates of the distributions.

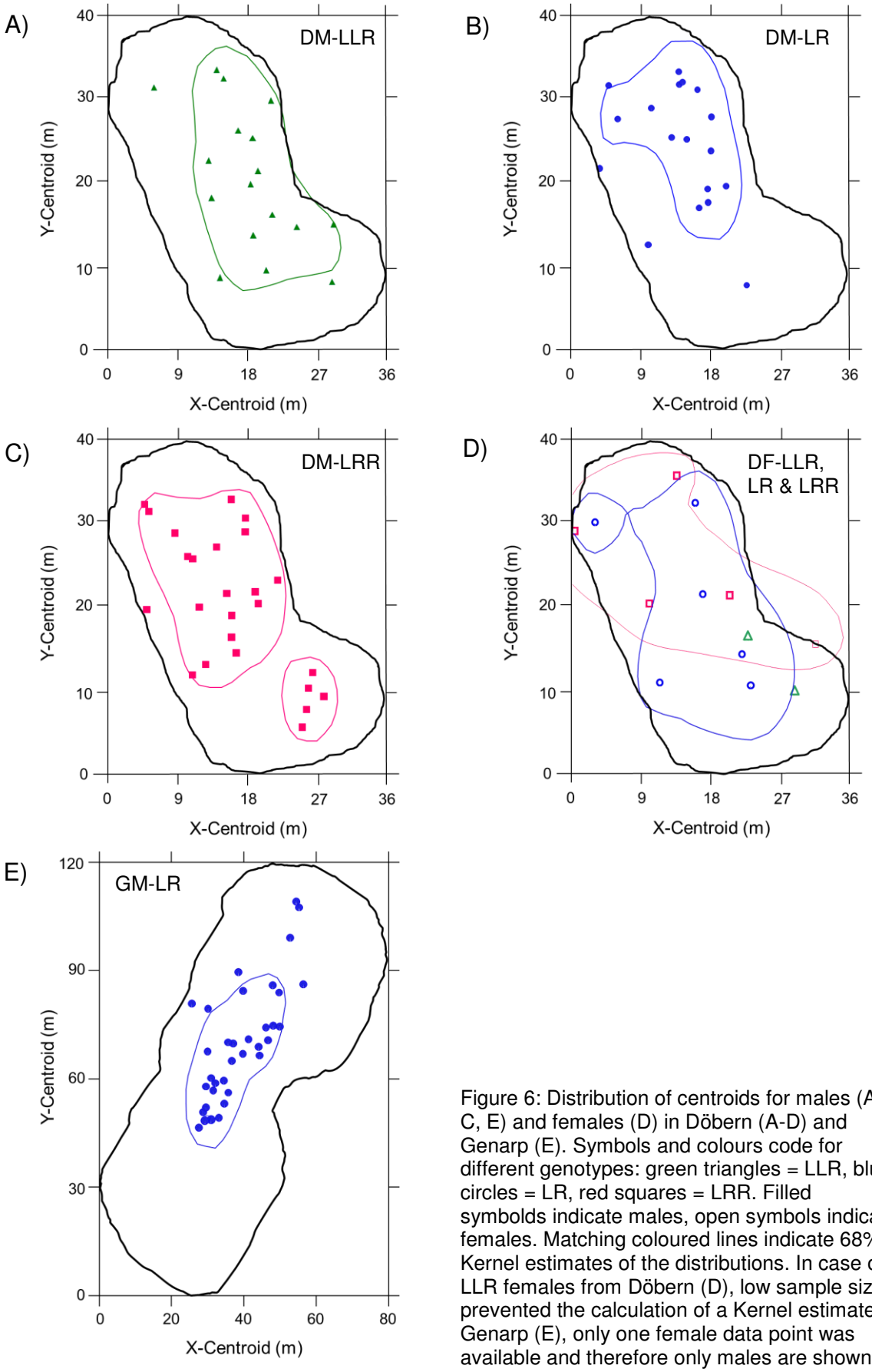

Figure 6: Distribution of centroids for males (A-C, E) and females (D) in Döbern (A-D) and Genarp (E). Symbols and colours code for different genotypes: green triangles = LLR, blue circles = LR, red squares = LRR. Filled symbolds indicate males, open symbols indicate females. Matching coloured lines indicate 68%-Kernel estimates of the distributions. In case of LLR females from Döbern (D), low sample size prevented the calculation of a Kernel estimate.In Genarp (E), only one female data point was available and therefore only males are shown.

Since the distribution of centroid localities showed that males were attracted to and aggregated in certain areas of the pond, we examined the distance of males to their nearest neighbor to investigate the small-scale patterns of their spatial distribution. As explained in the methods, we did not consider distances > 3m in the comparative analysis of nearest neighbor males, since large outliers might have obscured the possible influence of some variables and we did not consider larger nearest-neighbor distances relevant for small-scale interactions. Within a 3m radius, the average distance males kept to their nearest male neighbor was highest in Genarp (1.36 ± 0.59 m (1 S.D), n = 105), followed by Kloten (0.95 ± 0.50 m, n = 917) and Döbern (0.73 ± 0.54, n = 299). A GLM on all nearest-neighbor observations across the three populations and seven genotype combinations between nearest neighbor males showed that population indeed had the strongest influence on spatial distance. Further, the averaged body sizes of nearest neighbors had a significant positive effect, while the effects of average male condition and genotype were only marginal. The size of the difference in body size and difference in condition between nearest neighbor males did not play a role in the model (Table 3).

Table 5: GLM of distance to the nearest neighbor versus population, genotype combination, SVL (combined and differences) and BCI (combined and differences) . In the 'df'-column, (-) indicates variables that were eliminated during step-wise variable selection due to non-sufficient p-values. Significant p-values are printed in bold.

	DNN			
Source	df	coefficient	F-ratio	P
population	2		15.644	**< 0.0001**
AV_SVL	1	0.039	4.825	**0.028**
AV_BCI	-	-0.006	3.103	0.078
genotype	-		1.763	0.091
DIFF_SVL	-	0.043	2.266	0.133
DIFF_BCI	-	0.03	1.163	0.281
error	1260			

Reproductive behavior

From a total of 51 amplexus pairs (Kloten: 15, Döbern: 22, Genarp: 14) we determined the genotype of the involved male and compared it with the overall distribution of male genotypes in the population. In the two all-hybrid populations Döbern and Genarp, the number of amplexus males among genotypes did not differ from the overall distribution of the three genotypes within the population (Döbern: Chi^2=1.47, p=0.48, df=2; Genarp: Chi^2=0.61, p=0.74, df=2). In the Kloten population,

there was a tendency for *P. lessonae* males to be disproportionally more successful in clasping females into amplexus than *P. esculentus* males (Chi^2=3.131, p=0.077, df=1). We had measurements of distance variables for 27 out of 51 amplexus males (Kloten: 8 out of 15, Döbern: 12 out of 22, Genarp: 7 out of 14). When we performed logit regressions on this subsample of males separately for Kloten, Döbern and Genarp, we found that none of the considered independent variable (DTC, DBO, SVL and BCI) to contribute to amplexus success in males in any of the populations (Kloten: DTC: all $p \geq 0.190$, DBO: all $p \geq 0.205$; Döbern: DTC: all $p \geq 0.134$, DBO: all $p \geq 0.507$; Genarp DTC: all $p \geq 0.380$; DBO: all $p \geq 0.415$).

Discussion

No differences found between genotypes

The three ponds in our study showed considerable differences in size and population density. We therefore should infer and compare mating strategies between the sampled ponds with great caution, since mating systems and reproductive behaviors can show considerable plasticity among years and between populations due to environmental effects and population density (Kokko and Rankin 2006, Wells 2007). A prominent finding in our study was the inter-pond differences in spatial distance movement of individuals. Distance parameters between the two smallest ponds Kloten and Döbern were very similar, whereas individuals in the largest pond with the lowest population density (Genarp) moved significantly longer distances between subsequent observations than in the other ponds. We did not detect any spacing movement differences between *P. esculentus* and *P. lessonae* (L-E-population) in Kloten, nor between the three different genotypes of hybrid *P. esculentus* in Döbern (in Genarp we could not test for differences between genotypes). If we had detected patterns in male spatial behaviors among males from the E-E population in Döbern that could indicate differences correlated with a certain genotype, we would have attributed this to a genomic dosage effect, similar to the genomic composition-dependent variation in call characteristics that was found in some E-E populations (Hoffmann and Reyer 2013). Yet, our study did not yield any indication for genotype-dependent differences in distance movement behavior. This raises the question whether any genetic basis for differentiation of male reproductive behavior in all-hybrid populations of *P. esculentus* should exist. This question we could not answer

in our study, but since our analyses did not yield any differences between *P. lessonae* and *P. esculentus* males in Kloten, nor among different hybrid genotypes in the E-E system in Döbern, we suggest that *P. esculentus* movement does not substantially differ from *P. lessonae* and that spatial behavior in all-hybrid populations resemble the behavior observed in L-E-systems. Since we did not include systems involving male *P. ridibundus*, it remains an open question whether the behavioral similarities between males of *P. esculentus* and *P. ridibundus* observed in studies on R-E systems (Weidenberg 1999, Plötner 2001) result from an adaptation of the hybrid to a different population system involving *P. ridibundus* instead of *P. lessonae*. Since frogs in those studies originated from an R-E system, it is e.g. possible that females had different mating preferences than in studies on L-E systems, where females prefer the parental genotype (Abt and Reyer 1993, Roesli and Reyer 2000, Engeler and Reyer 2001). Female preferences in the R-E system have been studied less intensely than in the L-E system, but due to lower fertilization abilities of hybrid versus parental sperm (Reyer et al. 2003), a preference of *P. ridibundus* over *P. esculentus* males should yield a selection benefit to both parental and hybrid females.

Possible effects of density on DOB, DTC and DNN

While female choice and male competition are renownedly important factors for shaping animal mating patterns, Kokko and Rankin (2006) emphasize that density-dependent effects should not be neglected when studying mating systems. The three ponds in our study differed greatly in population density between Kloten (showing the highest population density) and Döbern/Genarp (relatively low density). Since the large differences we observed in spatial movement behavior between Genarp versus Kloten/Döbern were neither explained by genotype, body size nor body condition, we assume that these differences are likely to be density-dependent. While variation in population density affects the availability of mating partners for both sexes, this effect can be amplified for one sex in case of a biased operational sex ratio (Kokko and Rankin 2006). In the case of Kloten, high population density was paired with the lowest male/female ratio (1.26) of all populations, while the lower population densities in Döbern and Genarp were combined with a higher skew in their sex ratios (Döbern: 1.44, Genarp: 2.01), resulting in potentially low female encounter rates for individual males. We therefore suggest that competition among males was higher in the two all-

hybrid populations than in the L-E population in Kloten. However, the distribution of males and females was also related to relevant pond features. We found that males and females overlapped in the distribution of their movement centroids across all genotypes, but certain areas of the ponds were more frequented than others. These areas included structures like pond banks and vegetation types primarily suitable for hiding or ovipositing (females) and secondarily for sexual displays (males) and searching for a partner (both males and females). The more males there are relative to pond size, the more densely they should aggregate around spots that are frequented by females, and thus might have to compromise on individual distance. Consistent with this hypothesis, we found that male frogs in the Kloten and Döbern populations kept smaller nearest-neighbor distances than males in Genarp, which had a relatively low population density. Male frogs in dense aggregations can benefit from the proximity of other males. For one, they might indirectly profit from the enhanced signal function of a dense chorus on females, or profit directly if they manage to intercept an approaching female on the way to a more attractive neighbor. At very high densities, male spatial structure can even break down to a scramble-competition in populations that would otherwise show resource defense polygyny or lekking behavior if densities were lower (e.g. Grant et al. 1995, Byrne and Roberts 2004).

Effects of body size

In our study we observed significant differences in body size that were mostly related to genotype, but only between the smaller *P. lessonae* and larger *P. esculentus* males in Kloten. In the other two ponds, male genotypes generally overlapped too much to yield significant size differences, but there was a tendency among all-hybrid genotypes that LLR and LRR were larger than LR hybrids. While we did not detect any spatial patterns related to genotype, we found that the spatial distance between nearest male neighbors increases with average nearest-neighbor size. This means that big males keep at larger distance from other big males, while smaller males might be more likely to either be tolerated at closer range, or to fail in preventing their neighbors to come close to their own spot. Unfortunately, our data did not provide a causal relationship, but they clearly indicate some positive correlation between body size and nearest-neighbor distance among reproductively active males. This is supported by studies on toads, where larger males gain higher mating success than

smaller males by their mere physical dominance and ability to dislocate smaller males from amplexus (Davies and Halliday 1979). This can result in smaller males adopting alternative searching tactics, e.g. by searching in more peripheral areas and thus gaining access to females by intercepting them on their way to the pond (Forester and Thompson 1998). Although we did not quantify behaviors that could indicate such different tactics in our study, we found no indication that smaller males had limited access to females. Howard (1978) observed that mating behaviors in bullfrogs differed with male age and that older males were larger and defended higher-quality territories than younger males. Studying the sexual response of males from an L-E system towards females, Blankenhorn (1974) found that older *P. lessonae* males showed more sexual activity than younger males. We did not examine the age structure of our study specimens, but since size correlates with age within genotypes of water frogs (Blankenhorn 1974, Embrechts and Reyer 2012) body size should provide a good approximation of age. We assume that the largest individuals within each group of genotype were also the oldest and therefore possibly were more successful in defending their spot within a chorus against other males by keeping them at a larger distance. As spacing is also a consequence of mutual avoidance by calling males (Wells 1977), a male of large body size could trigger a stronger response (here: avoidance) among its competitors than a small male, irrespective of the competitor's own size. Body condition, on the other hand, did not seem to affect inter-male distance.

Proportional amplexus frequencies

When we examined which males actually gained amplexus opportunities, we did not find any evidence that size, condition or movement behavior of males influenced the propensity of being observed in amplexus. In the all-hybrid ponds, we did not find that the proportion of any genotype amongst amplexus males was higher than expected by their distribution. This is in accordance with an earlier study by Günther and Plötner (1990), who also accounted for the genotypes of females involved in amplexus, but did not find any sign of assortative mating in the all-hybrid population under study. In Kloten, we did observe a tendency of *P. lessonae* males to disproportionally prevail among amplexus pairs. In theory, assortative mating should occur in hybridogenetic water frog systems like the L-E system, when the dynamics of female preferences for parental males and the higher mating success of *P.*

lessonae males weigh heavier than the potentially higher primary fitness and aggression of hybrid males and thus functions as a stabilizing mechanism for the persistence of the system (Som et al. 2000). In practice, assortative mating favoring *P. lessonae* in L-E populations males has been demonstrated both in field and experimental studies (Blankenhorn 1974, Abt and Reyer 1993, Reyer et al. 1999, Roesli and Reyer 2000, Engeler and Reyer 2001, Abt Tietje 2003, Lengagne et al. 2006, Lengagne et al. 2008, Lengagne and Joly 2010), but the effect of assortative can be mitigated by a lack of preferred males or by intense male-male competition (Bergen et al. 1997).

Given the observed lack of differentiation in movement behavior between male genotypes in our study, spatial movement alone might not be an indicator of male mating success. This is confirmed by a study on movement of *Bufo americanus*, which found no evidence that movement correlates with mating success (Gatz 1981). In a study of an L-E-system in France by Lengagne and Joly (2010), the authors observed that the proportion of *P. lessonae* males distributed at “strategic” points at the edge of a chorus did not explain for their higher mating success. Rather, females exercised indirect paternity control by provoking male aggression and dislocation of the amplexing male when experimentally paired with an undesired (i.e. *P. esculentus*) male. When paired with a *P. lessonae* male, females usually accepted the male and behaved more cryptically towards competitors. Following this line of evidence that females can exercise paternal control mechanisms to get rid of undesired males, we could interpret the proportionally nondescript distribution of genome composition types in amplexus males among our two all-hybrid ponds as a lack of female choice between genome composition types. In a diploid-triploid system like the E-E system, a lack of female choice would be caused by the fact that a female preference for any genotype would be neither beneficial nor heritable, since triploid frogs stem from diploid parents and vice versa (Som and Reyer 2006). This hypothesis is experimentally supported by an study on *P. esculentus* from an E-E system in Sweden (in fact, from a locality close to the Genarp pond), where hybrid females did not show any preferences for male calls of either genome composition type in a choice experiment (Rondinelli 2006). Another explanation for a lack of female choice could be that female choice is limited by high male density and corresponding levels of background noise (Gerhardt and Klump 1988, Johnstone and Earn 1999), which affects female abilities to discriminate between competing males (Richardson and

Lengagne 2010). Indeed, experiments on other anuran species showed that females do not explicitly avoid less attractive males while approaching a male they actually prefer (Gerhardt et al. 1994).

Male-male competition and aggression

Another factor that might override assortative mating based on female choice in all-hybrid waterfrog populations are potentially high levels of aggression in hybrid males as a consequence of intense male-male competition. In general, vertebrate hybrids can be more aggressive than the parental species and have been shown to achieve higher reproductive success in some bird and fish hybrid complexes (McDonald et al. 2001, Rosenfield and Kodric-Brown 2003). Among European water frogs, direct comparison between *P. lessonae* and *P. esculentus* revealed that hybrid males tend to be more aggressive than their parental sympatrics (Lengagne et al. 2008). Although we did not focus on aggressive behavior in our study, we did observe aggressive behavior in some males that was even detrimental to the females they attempted to mate with: During the field work at the all-hybrid pond in Döbern, we frequently observed intense fighting over females and attempts to dislocate amplexus males by their competitors. We repeatedly observed dislocation of amplexing males from a female by other males, and the aggressive clasping sometimes grew so intense that several females suffered physical damage. For example, we captured three females that bore deep circular scarring from healed amplexus wounds (i.e. skin wounds caused by heavy clasping into the front leg pits) and AH saw one recently perished female with amplexus wounds so severe that parts of the intestines and ripe ovaries emerged from the body. In contrast, amplexus wounds were never observed in females from Kloten or Genarp. In Kloten, the relatively low male-female ratio and proportionally low number of hybrid males might have had a balancing effect on male aggression, and focal observations yielded only few physical aggressive encounters between males (H-UR, GAT). In Genarp, aggressive encounters between males appeared to be more ritualized and involved less direct physical contact, despite the fact that competition should have been intense due to a high male-female ratio. In a study on *Rana sylvatica*, Woolbright et al. (1990) observed that males were more active at high densities and suggested that males assess the density level of competitors and accordingly adjust their behavior. We assume that the low population density resulted in widespread roaming behavior and

larger inter-male distance among males in Genarp, which in consequence kept the general aggression level low in this population.

Conclusions

Our findings suggest that males of any genomic compositions in our study do not differ in their behavior based on genotype. Based on the apparently density-dependent movement behavior, spatial aggregation of movement centroids among individuals in all ponds and generally low inter-male distances, we could characterize male mating behavior in all three ponds as a type of lekking strategy where males aggregate and move around attractive spots and try to intercept females. For individual males this means moving around at close proximity to other males when population densitites are high, and spacing out at low densities in order to encounter dispersed females. Our study did not detect any differences in the spatial behavior of *P. lessonae* versus *P. esculentus* males, which means that any mating advantages of *P. lessonae* over the hybrid males might indeed be due to their higher inclination to grasp and mate with a female (Lengagne et al. 2006), especially at high population densities. Female choice in both *P. lessonae* and *P. ecsulentus* females has been shown to exist in several studies (Abt and Reyer 1993, Roesli and Reyer 2000, Engeler and Reyer 2001), but has been challenged by the male competition hypothesis by Lengagne et al. (2006), which claims that female choice should be overruled by male-male competition. We suggest that the key to the question which mechanism determines the formation of successful pairings among water frogs could be population density. For future studies we thus recommend an experimental approach on examining male competition and spacing behavior at varying densities.

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Long-term study of an infection with ranaviruses in a group of edible frogs (*Pelophylax kl. esculentus*) and partial characterization of two viruses based on four genomic regions

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Abstract

Several edible frogs (*Pelophylax kl. esculentus*) collected into a single group from various ponds in Europe died suddenly with reddening of the skin (legs, abdomen) and haemorrhages in the gastrointestinal tract. Ranavirus was detected in some of the dead frogs using PCR, and virus was also isolated in cell culture. Over the following 3 years, another two outbreaks occurred with low to high mortality in between asymptomatic periods. In the first 2 years, the same ranavirus was detected repeatedly, but a new ranavirus was isolated in association with the second mass-mortality event. The two different ranaviruses were characterized based on nucleotide sequences from four genomic regions, namely, major capsid protein, DNA polymerase, ribonucleoside diphosphate reductase alpha and beta subunit genes. The sequences showed slight variations to each other or GenBank entries and both clustered to the Rana esculenta virus (REV-like) clade in the phylogenetic analysis. Furthermore, a quiescent infection was demonstrated in two individuals. By comparing samples taken before and after transport and caging in groups it was possible to identify the pond of origin and a ranavirus was detected for the first time in wild amphibians in Germany.

Introduction

Ranaviruses are large (150–170 nm), icosahedral, double-stranded DNA viruses that belong to the family Iridoviridae. Since the first isolation of a ranavirus from *Lithobates pipiens* (formerly *Rana pipiens*) in 1965 (Granoff et al., 1965), an increasing number of infections caused by ranaviruses have been detected in ectothermic vertebrates (amphibians, fish and reptiles).

Although environmental changes are most likely to be the most important threat to amphibian populations, infectious diseases are suspected to play an important role in the global amphibian decline (Daszak et al., 1999). Most current studies focus on the fungus disease chytridiomycosis which has been termed the 'worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and it's propensity to drive them to extinction' (Gascon et al., 2007). However, disease caused by ranaviruses is also often associated with mass-mortality events and seems to occur worldwide (Gray et al., 2009). Ranaviral disease is therefore considered an emerging infectious disease in amphibians and is notifiable to the World Organisation for Animal Health (OIE).

In European amphibians, infections with ranaviruses have been detected in the UK in common frogs (*Rana temporia*) (Drury et al., 1995, Cunningham et al., 1996, Hyatt et al., 2000 and Duffus and Cunningham, 2010), common toads (*Bufo bufo*) (Hyatt et al., 2000 and Duffus and Cunningham, 2010), common midwife toads (*Alytes obstetricans*) (Duffus and Cunningham, 2010) and common or smooth newts (*Lissotriton vulgaris*, formerly *Triturus vulgaris*) (Duffus and Cunningham, 2010). The first proven ranavirus-associated mass-mortality event in mainland Europe occurred in Spain 2007 in common midwife toads (Balseiro et al., 2009). In connection with a second disease outbreak in the same species in the Spanish Pyrenees, a ranavirus was also detected in alpine newts (*Ichthyosaura alpestris cyreni*, formerly *Mesotriton alpestris cyreni*) (Balseiro et al., 2010). In Portugal, a ranavirus has been detected associated with mass mortality episodes affecting the newts *Triturus marmoratus* and *T. boscai* in 2003 (Alves de Matos et al., 2008).

The first report of a disease outbreak in *Pelophylax esculentus* (formerly *Rana esculenta*) in former Yugoslavia – described as 'viral haemorrhagic septicaemia of frogs', which probably resulted from ranavirus infection – was reported in 1968 (Kunst and Valpotic, 1968). Mass mortality events in this species caused by ranaviruses have also been detected in Croatia (Fijan et al., 1991), Denmark (Ariel et

al., 2009) and Italy (Ariel et al., 2010). In September 2010, the first ranavirus-associated mass mortality event in wild water frogs (*Pelophylax spp.*) and common newts occurred in the Netherlands (Kik et al., 2011). The virus found in that outbreak appears to be identical to the ranavirus (common midwife toad virus, CMTV) that was previously isolated in the Spanish Pyrenees (Balseiro et al., 2009 and Balseiro et al., 2010). The present study describes the detection and characterization of a ranavirus during an outbreak of fatal disease in a study group of edible frogs collected from various European ponds. Following the initial outbreak, surviving frogs were kept and sampled repeatedly for virus shedding. New animals were added to the group yearly. Screening was continued for 3 years and detected viruses were characterized based on partial sequences of four different genes.

Materials and methods

Outbreaks of disease

Adult frogs (4–5 years of age) of the *Pelophylax esculentus* complex were collected from wild populations for crossing experiments and behavioural studies on hybridization at the University of Zürich. In 2008, 218 frogs were collected from 17 localities (Table 1).

Table 1. Number of animals collected, original habitat, number of dead and surviving animals.

Year	Newly collected animals		Dead animals	Surviving animals
	Number	**Locality**		
2008	218	17 (ponds in Sweden, Czech Republic, Slovakia, Poland, Eastern Germany)	156	62
2009	97	9 (ponds in Sweden, Slovakia and Eastern Germany)	6 (collected 2008, died after hibernation); 4 (collected 2009, not clear whether or not they were infected)	56 (collected 2008); 93 (collected 2009)
2010	90	5 (ponds in Eastern Germany and Switzerland)	100 died 108 euthanased	23 (collected 2008); 93 (collected 2009)
2011	-	-	3 (died during hibernation)	28

Most frogs were exposed to handling during capture and transport, and were placed in restricted housing conditions in the laboratory during experiments for several days. After this time, the animals were kept under species-appropriate conditions in fenced outdoor enclosures (4 × 8 m2) containing an artificial pond with natural pond vegetation. Frogs were fed with crickets (*Acheta domestica*) and protected against predators by strong top netting.

Some days after the first release of frogs into the enclosures in late May, dead animals were detected. Pathological examination was performed on three of the affected frogs in June 2008. Prior to death, the animals showed no signs of pre-existing chronic disease conditions. Pathohistological changes were detected in several organs, namely, necrosis of lung capillaries partially associated with bacterial foci, minor haemorrhages, heterophilic infiltration and slight leukostasis, focal interstitial kidney necrosis associated with bacterial foci, multifocal bacteria in liver sinusoids, and multifocal necrosis of single liver cells. Oedema of the lamina propria including necrotic foci associated with bacteria was detected in the small intestine. Multifocal interstitial necrosis of the testis and multifocal bacteria in the vessels of the choroidea were also detected in one animal. Aeromonas sobria was isolated from the liver. Based on the morphological changes and bacterial results, a bacterial sepsis (‘red leg disease’) was diagnosed.

To prevent the spread of infection, apparently healthy animals were isolated (1–5 animals) in large cattle tanks (1.6 m × 1 m × 1 m) providing shelter and a pool of clean saline water (10 g NaCl/100 L aged water). An increase in water salinity was supposed to slow bacterial growth. Approximately 50 frogs were additionally treated with enrofloxacin corresponding to the antibiogram (bathing for 5 min in 1.5 mL/L H2O enrofloxacin 10% oral solution (Baytril, Bayer) for 5 days). However, this treatment did not seem to affect the progress of the disease since all frogs that showed symptoms at the beginning of the enrofloxacin treatment died.

Approximately 160 animals died in 2008. Signs of the disease included: haemorrhagic ulcerations of digits and joints (Fig. 1a), abnormal body shape (bloat due to oedema, cachexia), ventral petechial haemorrhages (‘red leg’) (Fig. 1b) and, rarely, hairy fungal plaques growing on skin. Some animals showed none of these symptoms, but morbidity was indicated by lethargic floating and impaired movement. Most sick animals died within 1 day after showing first signs of disease. The surviving frogs overwintered in small groups in plastic boxes with aged water and dry sitting

places in a cold room at 4–5 °C between November and March. Cleanness of water, room temperature and animal condition were checked on a regular basis. A very small number of the surviving frogs died during hibernation or soon after their release to the outdoor enclosures in spring 2009 (Table 1). The rest of the frogs remained healthy, and newly introduced animals did not show any signs of disease.

a.

b.

Fig. 1. a) Haemorrhagic ulcerations of digits and joints in a ranavirus infected edible frog (*Pelophylax kl. esculentus*). b) Ventral petechial haemorrhages on the lower abdomen and upper thighs of an edible frog (*Pelophylax kl. esculentus*) infected with a ranavirus.

In the summer of 2010, a second large disease outbreak occurred. The majority of the remaining group (108 apparently healthy animals in 2010, another 25 in 2011) were euthanased using an overdose of tricaine anaesthetic (buffered MS-222 solution 1 g/L) and stored at −20 °C for later examination. From a total of 405 frogs collected over 3 years, 277 died in connection with disease symptoms during three ranavirus outbreaks. A schematic timeline of outbreaks and testing of frogs is presented in Fig. 2. Release to the ponds of origin was not possible due to risk of infection.

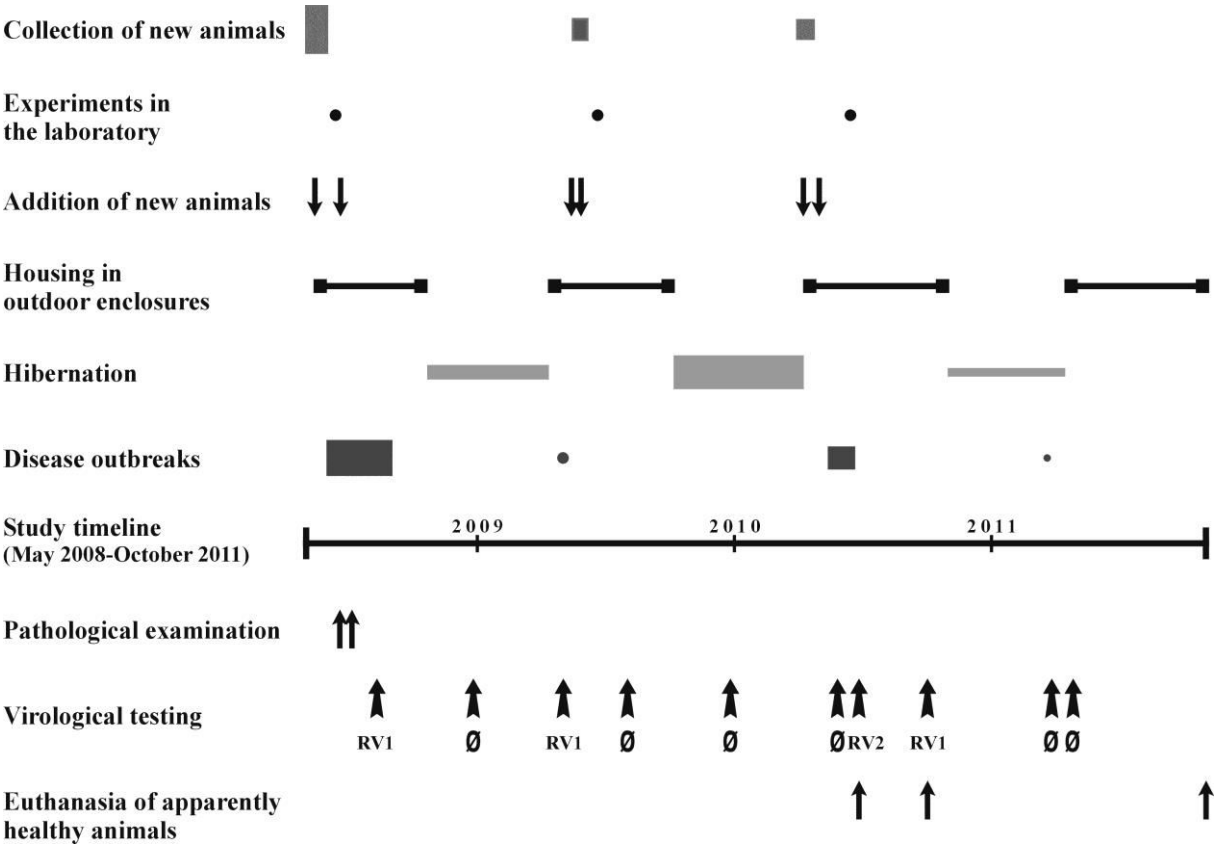

Fig. 2. Schematic timeline of outbreaks and testing of frogs. Box sizes are proportional to numbers of animals for the categories collection of animals, hibernation and disease outbreaks. Ø = no virus detected at sampling time point; RV1, Zuerich Pelophylax collection ranavirus 1; RV2, Zuerich Pelophylax collection ranavirus 2.

Sampling

Dead frogs frozen at −20 °C (2008, found dead: n = 8; 2009, found dead: n = 6; 2010, found dead: n = 27; 2010, euthanased: n = 9; 2011, found dead: n = 3) were sent for virological testing. Clinical signs and gross pathological changes are listed in Table 2. Histological examination of animals found dead was not undertaken, as the tissues were autolysed. Skin and cloacal swabs from asymptomatic frogs were

collected and submitted for virological testing (2008, n = 32; 2009 and 2010, n = 101; 2011, n = 30). In a retrospective study, toe clips from 229 frogs, which had been collected before removal from their habitat (2008–2010), and one ethanol-fixed edible frog that had died shortly after collection in 2008, were tested for the presence of ranaviral DNA.

Virus isolation

When it was possible to identify all organs of an animal (and depending on pathological findings) small tissue samples of the kidney, liver, intestine, spleen, heart and the skin were collected separately in cell culture medium; swabs and toe clips were individually collected in 3 mL Dulbecco's modified Eagle Medium (DMEM) (Biochrom) supplemented with antibiotics (penicillin-G solution 200 U/mL; streptomycin sulfate solution, 380 U/mL; gentamicin sulfate solution 6.4 U/mL; amphotericin B solution 0.5 µg/mL) (Biochrom).

After sonication and centrifugation at low speed (2000 g, 10 min), 200 µL of the supernatant was inoculated onto approximately 70% confluent iguana heart cell monolayers (IgH-2, ATCC: CCL-108) in 30 mm diameter tissue culture dishes (Cellstar, Greiner Bio-One). After incubating for 2 h at 28 °C, each dish was cultured with 2 mL nutrient medium (DMEM supplemented with 2% fetal calf serum (FCS) (Biochrom) and 1% non-essential amino acids (NEA; Biochrom). Tissue cultures were observed twice a week for cytopathic effects (CPE). When a CPE appeared, the cultures were frozen at −20 °C, thawed and reinoculated onto IgH-2 for a second passage. Dishes showing no CPE were frozen after 2 weeks incubation for a second passage.

Polymerase chain reaction, sequence analysis

DNA was extracted from the original sample or from cell culture supernatant using the DNeasy Kit (Qiagen), and PCR was undertaken for the detection of ranaviruses in 25 µL reaction tubes as described previously (Mao et al., 1997 and Marschang et al., 1999) using primers OL T1 and OL T2R targeting a 500 bp portion of the ranavirus major capsid protein (MCP) gene.

Table 2. Samples from a group of *Pelophylax* kl. *esculentus* analyzed over the course of 3 years with short case histories, the results of virus isolation on cell culture, PCR and sequencing.

Date of sampling	Case history	Clinical signs	Sample type	Number of samples	Virus isolation	PCR from original sample	Sequencing, type of Virus
06-07/2008	High mortality, abnormal body shape (bloat due to oedema, anorexia), lethargic floating and impaired movement	Reddening of the skin (legs, abdomen), rarely: hairy fungal plaques growing on skin, haemorrhages in the gastrointestinal tract, fragile intestine, brown aqueous ascites	Frozen animals (kidney, liver, intestine, spleen, heart)	8	7/8 positive (kidney: 6/8 liver: 6/8 intestine: 5/8 spleen: 5/8 heart: 7/8)	7/8 positive (kidney: 7/8 liver: 6/8 intestine: 6/8 spleen: 7/8 heart: 5/8)	Isolates from three animals sequenced (ZPRV1)
12/2008			Skin swabs	32	-	-	-
04/2009	Several animals died during artificial winter or shortly after removal to outdoor enclosures	Most animals: reddening of the skin (legs, abdomen); one animal: ascites, partially dark red coloured intestine, renomegaly	Animals (kidney, liver)	6	-	3/6 positive (kidney: 3/6 liver 3/6)	n.d.
04/2009			Skin + cloacal swabs	19	4/19 positive (skin swabs: 3/19 cloacal swabs: 2/14)	14/19 positive (skin swabs: 14/19 cloacal swabs: 10/19)	Isolates from two animals sequenced, partial sequences (MCP, DNApol) from two animals (ZPRV1)
07/2009			Skin + cloacal swabs	30	-	-	-
12/2009			Skin + cloacal swabs	28	-	-	-
05/2010			Skin + cloacal swabs	24	-	-	-
05-06/2010	High mortality or euthanasia of apparently healthy animals	Some dead animals: reddening of the skin, locomotive troubles	Animals (kidney, liver)	32	23/32 positive (kidney + liver: 21/32, only kidney: 1, only liver:1)	22/32 positive (kidney: 19/32 liver: 21/32)	Isolates from two animals sequenced (ZPRV2)
09/2010	Euthanasia	Reddening of the skin; one animal: hepatomegaly, splenomegaly	Animals (kidney, liver, spleen)	4	2/4 positive (kidney: 1/4, spleen 1/1)	-	Isolates from two animals sequenced, (ZPRV1)
03/2011	Animals found dead in their cages during artificial winter	red fluid ascites, haemorrhages in the kidneys, fragile yellow liver with small dark dots	Animals (kidney, liver, skin)	3	-	-	
04/2011			Skin swabs	30	-	-	

For positive tested samples, additional PCRs targeting the major part (1402 bp) of the MCP gene in overlapping fragments, partial sequences of the DNA polymerase (DNApol), ribonucleoside diphosphate reductase beta subunit-like protein (RNR-α) and alpha subunit-like protein (RNR-β) genes were performed using different primer pairs for each gene (Table 3). Primers and reaction conditions have been published before (Ariel et al., 2010, Hyatt et al., 2000 and Holopainen et al., 2009). Oligonucleotides were purchased from MWG Biotech. The obtained PCR products were separated by agarose gel electrophoresis (1.5% agarose gel (Bioenzym) in TAE buffer containing 0.5 µg/mL ethidium-bromide and evaluated under 320 nm UV light. PCR amplicons were gel purified using peqGOLD gel extraction kit (Peqlab Biotechnologie) and sent for sequencing from both directions to MWG Biotech.

Table 3. Primers used in PCR reactions.

Target gene	Primer	Primer position	Amplicon size	Nucleotide sequence (5' to 3')	Reference
MCP	OL-T1 OL-T2R	97387-97404 97917-97899	531	GACTTGGCCACTTATGAC GTCTCTGGAGAAGAAGAAT	Mao et al. (1997); Marschang et al. (1999)
	MCP-BF MCP-BR	97813-97830 98360-98341	548	ACCAGCGATCTCATCAAC AGCGCTGGCTCCAGGACCGT	Ariel et al. (2010)
	MCP-6 MCP-6R	98244-98263 98828-98807	585	CGCAGTCAAGGCCTTGATGT AAAGACCCGTTTTGCAGCAAAC	Hyatt et al. (2000)
DNApol	DNApol-F DNApol-R	67188-67208 67747-67728	560	GTGTAYCAGTGGTTTTGCGAC TCGTCTCCGGGYCTGTCTTT	Holopainen et al. (2009)
RNR-α	RNR-AF RNR-AR	43729-43748 44534-44513	806	CTGCCCATCTCKTGCTTTCT CTGGCCCASCCCATKGCGCCCA	Ariel et al. (2010)
RNR-β	RNR-BF RNR-BR	78029-78012 77384-77403	646	AGGTGTRCCRGGGYCGTA GACGCTCCAYTCGACCACTT	Ariel et al. (2010)

The primer position is presented relative to the FV3 genome (AY548484).
Y = C/T, K = G/T, S = C/G, R= A/G

The sequences were edited, assembled and compared using STADEN Package version 2003.0 Pregap4 and Gap4 programmes (Bonfield et al., 1995). The edited original sequences were compared to those in GenBank online1 using BLASTX and BLASTN. Multiple alignments of nucleotide sequences were performed with the ClustalW algorithm of the BioEdit Sequence Alignment Editor program (Hall, 1999). This alignment was further used for phylogenetic calculations in the PHYLIP program Package version 3.6. (Felsenstein, 1989) trying distance based, maximum-likelihood and parsimony methods to obtain an optimal tree. Bootstrap analysis of 100 replicates was carried out. GTR + G (general time reversible assuming gamma distribution) substitution model for MrBayes (with 1 million generations, sample frequency: 10 and burnin ratio: 40%) was also used to reconstruct phylogenies (Huelsenbeck and Ronquist, 2001) as an application of the TOPALi v2.5 programme.

Results

During suddenly increased mortality in the summer of 2008, ranavirus was detected by MCP gene PCR in 7/8 tested edible frogs (Table 2) and isolated in cell culture from the same seven animals. Twenty-eight per cent of the animals from the infected group in Zürich survived the outbreak and did not show any symptoms during the following months. Before hibernation of the surviving animals, no virus was detected in skin swabs (n = 32), but six animals died during or shortly after artificial winter, three of which were tested positive for ranavirus in liver and kidneys via PCR. Several days after this second outbreak, skin and cloacal swabs from 19 apparently healthy animals were taken and ranavirus was detected in 14 frogs via PCR, virus was isolated in cell culture from four animals. Until May 2010, none of the remaining animals showed any signs of disease and virological testing from a total of 83 skin and cloacal swabs was negative.

Some days after new edible frogs were added, a third disease outbreak with high mortality occurred in May 2010. A total of 22/32 examined frogs tested positive for ranavirus by PCR (Table 2) and virus was isolated from 23 animals. No virus was detected in the apparently healthy animals which were euthanased during this outbreak whereas all examined animals which died naturally were tested positive. For further characterization additional gene sequences of the obtained isolates from each outbreak were analyzed as described previously (Ariel et al., 2010, Hyatt et al., 2000 and Holopainen et al., 2009) (Table 3). These studies showed the presence of

two distinct ranaviruses in this group of animals – one from the first outbreak to the end of the study (Zuerich Pelophylax collection ranavirus 1, ZPRV1), the other in association with the third outbreak in May 2010 (ZPRV2) (Fig. 2). Sequences from multiple isolates of each of the two viruses were always identical (Table 2). While the two different viruses showed high similarity to each other in the nucleotide sequences of the partial sequences from the MCP, DNApol and RNR-α subunit genes, the partial sequences from the RNR-β subunit gene were 100% identical to one another. Comparison of the amino acid sequences showed that all differences except those on the RNR-α subunit were silent mutations. Comparison of the sequences of the viruses detected in this group of frogs with corresponding sequences from the FV3 genome showed identities of 98–99% (Table 4).

Table 4. Ranavirus (RV) sequence identity of the four analyzed parts of the genome. The two different ranaviruses (ZPRV1 and 2) detected in this study are presented in comparison to FV3. For each gene sequence, the upper diagonal shows the values for the nucleotide sequence identity, the amino acid identity values are provided in the lower diagonal.

MCP	ZPRV1	ZPRV2	FV3
ZPRV1		99.79%	98.07%
ZPRV2	100%		98.15%
FV3	97.74%	97.74%	

DNApol	ZPRV1	ZPRV2	FV3
ZPRV1		99.81%	98.84%
ZPRV2	100%		99.04%
FV3	98.27%	98.27%	

RNR-α	ZPRV1	ZPRV2	FV3
ZPRV1		99.74%	98.82%
ZPRV2	99.21%		98.82%
FV3	98.82%	98.82%	

RNR-β	ZPRV1	ZPRV2	FV3
ZPRV1		100%	98.68%
ZPRV2	100%		98.68%
FV3	98.51%	98.51%	

GenBank accession number for FV3: see Table 3

In the phylogenetic analysis, the gene sequences of each of the two viruses (ZPRV1, ZPRV2) were concatenated (3223 bp) and studied in comparison to previously published ranavirus sequences available in GenBank from amphibians, fish, and a reptile. Both viruses (ZPRV1 and 2) clustered closely to each other and to the Rana esculenta virus (REV-like) clade (Fig. 3). The obtained gene sequences of each virus (ZPRV1 and 2) were submitted to GenBank with accession numbers KC440841, KC440842 (MCP), KC440843, KC440844 (RNR-α), KC440845 (RNR-β), KC440846, KC440847 (DNApol).

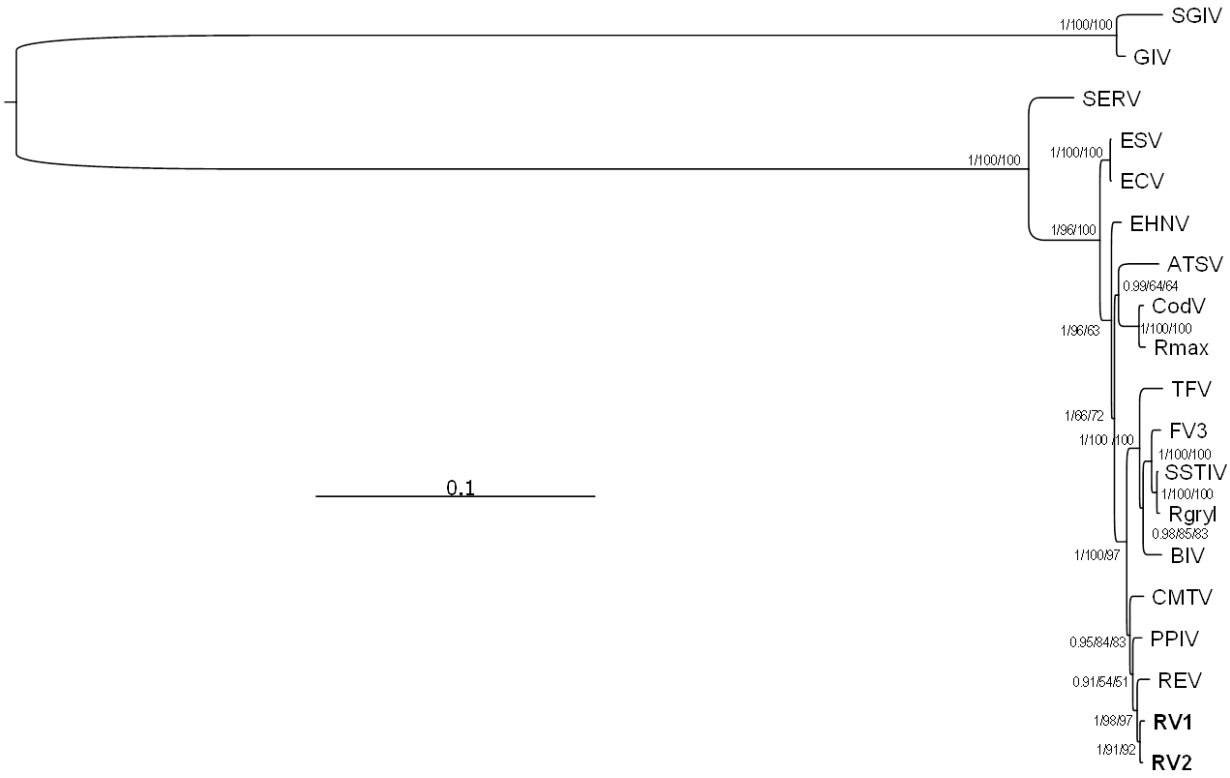

Fig. 3. Midpoint rooted MrBayes tree of the concatenated nucleotide sequences

In September 2010, the previously detected ranavirus (ZPRV1) was discovered in 2/4 euthanased individuals. In one animal, splenomegaly was observed and virus was isolated only from the spleen, the other animal showed no pathological changes and virus was isolated from the kidneys. Testing of tissues from these animals (liver, kidneys and spleen) via PCR was negative. Interestingly, both animals were collected in 2008 and tested positive only by PCR in April 2009 (skin and cloacal swabs) but never developed clinical disease. Sequencing of a part of the MCP and the DNApol gene from the previously tested swabs (from 2009) demonstrated that

this ranavirus was 100% identical to the isolates obtained in September 2010 (ZPRV1). No ranavirus could be detected in three animals which died during hibernation 2011 and skin swabs from 30 frogs tested negative in April 2011.

In order to determine from which pond the infection was originally introduced, a total of 229 available pre-transport DNA samples were screened in 2008 (all ponds), 2009 (four ponds) and 2010 (one pond). Ranavirus was detected in a single sample from 2008. This animal died shortly after removal from its habitat (Untermassfeld, Germany) and was fixed in ethanol. By repeating testing using skin from the fixed animal, we were able to verify the infection. The virus detected was identical to the obtained isolates from 2008 and 2009 (ZPRV1). The origin of ZPRV2 could not be identified.

Discussion

Two different manifestations of ranaviral disease have been described in European amphibians, namely, an acute, systemic haemorrhagic disease and a cutaneous form (ulcerative syndrome) which seems to be more chronic (reviewed in Duffus and Cunningham, 2010). In our cases, the symptoms of the diseased animals varied, so they could not be clearly correlated to one of the proposed forms of disease. The documented bacterial co-infection in combination with the stress of transport are likely to have influenced the course of disease as described, for example, in an American bullfrog (Rana catesbeiana) ranaculture facility where >50% mortality and related pathological findings occurred due to a co-infection with a ranavirus and Aeromonas hydrophila (Miller et al., 2007).

The second disease outbreak with low mortality in spring 2009 was associated with relatively low environmental temperatures. Previous investigations have demonstrated the dependency of ranavirus replication on temperature (Rojas et al., 2005). Several authors have suggested that the amphibian host immune function decreases at lower temperatures (Maniero and Carey, 1997, Carey et al., 1999, Forbes et al., 2004, Rojas et al., 2005 and Raffel et al., 2006) and pathogen infectivity can therefore increase. Translocation after hibernation may also have influenced the susceptibility of the immunocompromised animals to disease.

Interestingly, PCR seemed to be more sensitive than cell culture in detecting ranavirus in skin or cloacal swabs in April 2009. Previous studies demonstrated that non-lethal sampling techniques are useful for ranavirus diagnostics, but the

prevalence of infection may be underestimated in comparison to liver samples (Gray et al., 2012). It is questionable whether PCR was able to detect very low amounts of replication competent virus, or if viral DNA instead of active virus was detected. It is also possible that the virus was only on the surface of the skin or the cloaca without infecting the animal. By infecting Xenopus laevis with FV3 via water, Robert et al. (2011) demonstrated that FV3 was transcribed in the skin of only a few frogs and suggested that despite the presence of virus on the skin surface, little or no virus replication was initiated at an early stage of infection. Nevertheless, our findings could be interesting in defining the best time to screen live animals for an infection by PCR as the animals seemed to release detectable amounts of virus into the environment after hibernation and in the breeding season.

A number of studies have been carried out to understand the amphibian adaptive and innate immune response to ranavirus infections, mostly using the Xenopus model (see, for example, Gantress et al., 2003, Robert et al., 2005, Maniero et al., 2006 and Morales et al., 2010). It has been shown that animals are able to clear ranavirus infection and that after reinfection with the same virus viral clearance was markedly accelerated and animals did not show any symptoms of illness (Gantress et al., 2003). As our two isolated viruses showed only slight variations to each other on the characterized genes, it is remarkable that not only newly added animals died in 2010, but also that animals which had been in contact with ranavirus previously were not able to resist the infection. It is possible that the new virus strain was more virulent or that the immune response was not able to clear the infection as the antibodies may have weak affinity (Maniero et al., 2006). Another factor might be an immunocompromised state of the new co-housed animals.

We hypothesize that the two frogs in which the first ranavirus (ZPRV1) was detected following euthanasia in September 2010 were infected with the ZPRV1 in 2008 but did not develop disease due to an effective antiviral immune response. Nevertheless, the infection was not eliminated completely and the animals appear to have harboured quiescent virus over a period of at least 1 year. Quiescent infections have been shown to occur in X. laevis, in which ranaviruses can remain in peritoneal leukocytes (Robert et al., 2007 and Morales et al., 2010). It is possible that the frogs were shedding ranavirus at the time of sampling in April 2009. On the other hand, no virus was detected in two other euthanased frogs which were collected in 2008 and

also tested positive in April 2009. These animals seem to have successfully cleared the infection.

Due to the retrospective nature of our study, we were able to detect ranavirus in a wild amphibian in Germany for the first time. It is possible that the positive tested animal or another animal from the same habitat first infected the group; the second type of ranavirus was probably introduced with newly collected animals in 2010, potentially from one of the ponds that we were not able to screen for ranaviral DNA in original toeclips. No mass mortality event was reported in any of the ponds in 2009 and 2010. As only one frog of those examined from their original habitat tested positive, it is possible that several samples tested were false negatives. It is also possible that the methods we used were not sensitive enough to detect small amounts of ranavirus in fixed toe clips or that storage of the DNA over a long period may have influenced the results.

Previous studies have demonstrated variations among different amphibian species to disease and variations in virulence between different virus strains (see, for example, Schock et al., 2008 and Hoverman et al., 2011). The global trade in amphibians, such as the translocation of larval tiger salamanders (Ambystoma tigrinum) as fishing bait or the commercial exploitation of Xenopus for research and as pets, is an important source of pathogen pollution (Robert et al., 2007 and Picco and Collins, 2008). Our report underlines the risks not only of introducing animals into new habitats but also of mixing amphibians from different origins, even when the animals appear clinically healthy.

Virus characterization based on partial genome sequencing is an important tool in understanding the course of ranaviral disease. Results of sequencing also allowed us to identify at least one source of infection. As the MCP gene is highly conserved, a part of it is very useful for diagnostics. To differentiate between various virus strains, sequence information from more genes is necessary. In the phylogenetic analysis, both ranavirus isolates detected during this study (ZPRV1, ZPRV2) were most closely related to each other. Interestingly, they also clustered close to other European isolates from amphibians and fish (REV, CMTV and PPIV) (Fig. 3). Additional analyses are necessary to help understand the capacity of ranaviruses to adapt to new hosts, their phylogenetic relationships, variations in virulence among species and between different ranavirus strains.

Conclusions

Two different ranaviruses have been identified as causative agents for recurring disease outbreaks with low to high mortality in edible frogs collected from multiple ponds in Europe to form a single group. It has been shown that animals can be sublethally infected and harbour quiescent virus over a period of at least 1 year. Co-housing of apparently healthy animals after capture and translocation should therefore be avoided. In addition, a ranavirus was detected in a wild amphibian from Germany for the first time. In the phylogenetic analysis, both ranaviruses detected in this study were most closely related to each other and to other European ranavirus isolates from amphibians and fish.

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In memoriam Lezcek Berger († 2012).

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